REMARKS

Applicant respectfully requests reconsideration. Claims 1-5, 8-20, 22, 27-32, 43, 45-57, 63-65, 70-73, 76-80, 83, 84, 88, 89, 94, 95, 97 and 99 are pending in this application with claim 1 being an independent claim. Claims 5, 13, 15, 45-57, 63-65, 70-73, 76-80, 83-84, 88-89, 94-95 and 97 are currently withdrawn. Upon allowance of claim 1, reconsideration and in some cases rejoinder of the withdrawn claims is requested.

Double Patenting Rejection

The Examiner has provisionally rejected claims 1-4, 8-11, 16-20, 22, 27-32, 43 and 99 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 41-46, 52-56 and 58-60 of co-pending Application No. 10/816,220. As stated in the previous response, Applicant defers substantive rebuttal of the provisional rejection until the cited co-pending application is allowed, and in the alternative requests withdrawal of the rejection once it is the only remaining rejection in the instant case. Applicant further notes that there is no outstanding rejection against claims 2, 3, 8-11, 16-20, 22, 27-32, 43 and 99, and therefore such claims should be deemed allowed and the double patenting rejection with respect to these claims should be withdrawn.

Rejection under 35 U.S.C. §112, enablement

Claims 1, 4, 12, and 14 stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. Applicant respectfully traverses for the reasons set forth below.

Applicant has previously presented a full Wands analysis relating to the enablement of the pending claims. Applicant will not re-iterate that analysis, but will instead rebut the issues raised by the Examiner in the instant Office Action.

Claim 1 and claims dependent on claim 1 relate to a composition comprising an immunostimulatory nucleic acid comprising the nucleotide sequence of SEQ ID NO:1. The test of enablement is whether one of ordinary skill in the art can practice (i.e., make and use) the claimed invention without undue experimentation. The Examiner acknowledges that the claimed composition can be made. However, the Examiner states that the composition cannot be used

because its use is limited to the treatment and/or prevention of cancer in a human subject, and Applicant has not demonstrated such use. Applicant respectfully traverses.

MPEP 2164.01(c) states that "when a compound or composition is not limited by a recited use, any enabled use that would *reasonably correlate* with the entire scope of that claim is sufficient to preclude a rejection for nonenablement based on how to use" (emphasis added). The specification clearly states that one use of the claimed composition, including the recited nucleic acid, is immunostimulation. For example, the specification teaches that the claimed composition can be used to induce and/or enhance immune responses, including innate immune responses, antigen-specific immune responses, and mucosal and systemic immune responses. The claimed compositions can be used with antigens for vaccination purposes, or for immunotherapy or general enhancement of immune function. The specification teaches that one goal of immunotherapy is to augment an immune response to an established tumor. Immunotherapeutics such as anti-cancer antibodies can be used together with the claimed compositions in order to stimulate antibody-dependent cell cytotoxicity (ADCC). The specification further teaches the claimed composition can be used to stimulate antibody production in vivo, including anti-cancer antibody production. Accordingly, the specification provides a number of uses for the claimed composition, and all of these uses reasonably correlate with the scope of claims 1, 4, 12 and 14.

The Examiner however rejects immunostimulation as not reasonably correlating with the currently elected species recited in claims 4, 12 and 14. Claim 4 recites that the composition further comprises an antigen such as a cancer antigen. Claim 12 recites that the composition further comprises a therapeutic agent such as an anti-cancer agent. Claim 14 further limits the type of anti-cancer agent. None of the additional constituents of claim 4, 12 and 14 precludes the use of the claimed nucleic acid as an immunostimulant. Rather, each takes advantage of the immunostimulatory properties of the claimed nucleic acid. For example, the antigen of claim 4 can be used together with an immunostimulatory agent in order to further promote an immune response and in particular an antigen-specific immune response. To this end, Applicant has demonstrated in Example 2 and FIGs. 15 and 16 the ability of a nucleic acid consisting of SEQ ID NO:1 to stimulate an antigen-specific immune response when administered to a murine subject together with a microbial antigen. The Examiner provides no reasonable rationale for

why the combination of the same nucleic acid with a cancer antigen would not also yield an antigen-specific immune response in the same system. Similarly, the anti-cancer medicaments of claims 12 and 14 can be used together with an immunostimulatory agent in order to further promote an immune response. One of ordinary skill in the art will recognize that an immunotherapeutic such as an anti-cancer antibody can be used together with the claimed compositions to induce or enhance ADCC. Similarly, one of ordinary skill in the art will recognize that a chemotherapeutic agent that is able to kill cancer cells will help generate cancer cell debris including cancer antigens. The presence of an immunostimulatory agent in such an environment will induce an immune response to the released cellular debris, including any released cancer antigens. Therefore, induction of an immune response reasonably correlates with the combined use of the claimed immunostimulatory nucleic acids with antigens or anti-cancer agents.

The Examiner maintains that the only use that correlates with the entire scope of claims 4, 12 and 14 is treatment and/or prevention of cancer in human subjects. In other words, the Examiner is requiring in vivo human data to support these claims, and then only in the form of evidence of treatment and/or prevention of cancer. Such evidence can only be gotten via human clinical trials in accordance with the FDA. The Patent Office has never required human clinical trial data as a prerequisite for patentability. In re Brana 51 F.3d 1560 (Fed. Cir. 1995) The unduly burdensome standard asserted by the Examiner is not supported by the case law.

As stated in the specification, the invention was based on the discovery of a nucleic acid sequence (SEQ ID NO:1) having an immune stimulation profile in vitro and in vivo similar and in some respects better than a previously known CpG nucleic acid (i.e., CpG nucleic acid 7909). The claimed invention relates to nucleic acids that minimally comprise SEQ ID NO:1, rather than a genus of sequence unrelated nucleic acids. Still many of the Examiner's comments are directed to the entire genus of CpG nucleic acids, with apparent disregard to the fact that the claims are directed to nucleic acids sharing a 24-mer consensus sequence to which immunostimulatory activity is attributed.

Applicant sought a nucleic acid having activity similar to or better than CpG 7909 at least because CpG 7909 was a preferred clinical candidate compound showing in vivo immunostimulatory effects, including in cancer subjects. Approximately 165 nucleic acids were

screened in order to identify the claimed 24-mer sequence. CpG 7909 (5' TCG TCG TTT TGT CGT TTT GTC GTT 3') is similar to the claimed nucleic acids: both sequences contain 4 CpG motifs and at least 2 regions comprising at least 4 thymidines. Importantly, the nucleic acids are functionally similar. The nucleic acids are able to (a) stimulate proliferation and activation of human B cells in vitro to similar levels as shown in FIGs. 1 and 6; (b) stimulate secretion of IFN-alpha, IP-10 and IL-10 from human cells to similar levels as shown in FIGs. 2-4 and 8-10; (c) induce TNF alpha from human cells to similarly low levels as shown in FIG. 11; (d) induce IL-12, IL-6 and TNF alpha secretion in mouse splenocytes to similar levels as shown in FIG. 13; (e) induce mouse B cell proliferation to similar levels as shown in FIG. 12; (f) enhance NK lytic activity in mouse splenocytes to similar levels as shown in FIG. 14; (g) enhance antibody production (and thus titer) against HBsAg in a murine in vivo model system as shown in FIG. 15); and (h) induce a Th1 biased immune response as evidenced by similar ratios of IgG1 vs. IgG2 in mice challenged with HBsAg. Many of these immune responses correlate with in vivo immune response induction, particularly in the context of cancer. See for example Weiner et al (J. Leuk. Biol. 2000 68:456-463) which documents the ability of CpG nucleic acids that induce B and NK cell activation, antigen-specific antibody production, and IP-10, IL-10 and IFN-alpha secretion to induce immune responses in murine tumor models. Moreover, as documented in the references cited in the previous responses, CpG 7909 induces immune responses in human cancer subjects. For example, van Ojik et al. (Ann. Oncol. 2003 13:157) demonstrates induction of an antigen-specific immune response when CpG 7909 is administered with the MAGE-3 cancer antigen, and Speiser et al. (J. Clin. Invest. 2005 115:739-746) demonstrates induction of an antigen-specific immune response when CpG 7909 is administered with the melanoma A cancer antigen. These references are submitted herewith for the Examiner's consideration.

Accordingly, the evidence made of record by the Examiner and Applicant establishes a correlation between the in vitro and in vivo data presented in the specification and immunostimulatory effects in vivo, including in cancer subjects.

The Examiner has not provided any reasonable basis for why these findings when taken together with the similar in vitro and mouse in vivo profiles of the claimed nucleic acids and CpG 7909 are not sufficient to establish use of the claimed nucleic acids in immunostimulation, whether used alone or with an antigen or an anti-cancer agent.

In view of the foregoing, withdrawal of the rejection of claim 1, 4, 12, and 14 under 35 U.S.C. §112 is respectfully requested.

CONCLUSION

A Notice of Allowance is respectfully requested. In the event this response does not place the application in condition for allowance, Applicant requests an interview with the Examiner.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

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Respectfully submitted,

By _____

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Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909

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The induction of potent CD8+ T cell responses by vaccines to fight microbes or tumors remains a major challenge, as many candidates for human vaccines have proved to be poorly immunogenic. Deoxycytidyl-deoxyguanosin oligodeoxynucleotides (CpG ODNs) trigger Toll-like receptor 9, resulting in dendritic cell maturation that can enhance immunogenicity of peptide-based vaccines in mice. We tested whether a synthetic ODN, CpG 7909, could improve human tumor antigen–specific CD8+ T cell responses. Eight HLA-A2+ melanoma patients received 4 monthly vaccinations of low-dose CpG 7909 mixed with melanoma antigen A (Melan-A; identical to MART-1) analog peptide and incomplete Freund's adjuvant. All patients exhibited rapid and strong antigen-specific T cell responses: the frequency of Melan-A–specific T cells reached over 3% of circulating CD8+ T cells. This was one order of magnitude higher than the frequency seen in 8 control patients treated similarly but without CpG and 1–3 orders of magnitude higher than that seen in previous studies with synthetic vaccines. The enhanced T cell populations consisted primarily of effector memory cells, which in part secreted IFN-γ and expressed granzyme B and perforin ex vivo. In vitro, T cell clones recognized and killed melanoma cells in an antigen-specific manner. Thus, CpG 7909 is an efficient vaccine adjuvant that promotes strong antigen-specific CD8+ T cell responses in humans.

Introduction

A major goal of therapeutic cancer vaccines is the induction of large numbers of antigen-specific T cell populations with effector functions that are able to mediate immune protection. In contrast to viruses and other pathogens, vaccines containing recombinant proteins or synthetic antigenic peptides usually fail to induce significant immune responses unless they are mixed with adjuvants (1, 2). Effective adjuvants display at least 2 mechanisms of action: a depot effect that leads to prolonged antigen exposure in the host, and a capacity to trigger the innate immune system through activation of DCs via Toll-like receptors (TLRs) (3–5). Upon proper antigen presentation, activated DCs play a key role in the induction of T cell responses (6). Because of their high efficacy, several recently identified TLR ligands are promising vaccine adjuvants.

Synthetic deoxycytidyl-deoxyguanosin oligodeoxynucleotides (CpG ODNs) contain unmethylated CG motifs similar to those observed in bacterial DNA. CpG ODNs elicit a complex immunomodulatory cascade that includes the production of T helper-1–type cells and proinflammatory cytokines (7). CpG ODNs directly stimulate DC activation through TLR9 triggering (8, 9), leading to enhanced T cell responses specific for coadministered antigens in mice (10–14). For example, we have reported previously that addition of CpG ODNs to melanoma antigen A_{26-35} peptide (Melan-A_{26-35} peptide; a widely used antigenic peptide in vaccine trials of HLA-A2+ melanoma patients) mixed with incomplete Freund's adjuvant (IFA) increased Melan-A–specific T cell responses in HLA-A2 transgenic mice (15). However, the CpG motifs that stimulate the murine immune system are suboptimal for stimulating the human one. Indeed, TLR9-expressing human cells are susceptible to distinct CpG motifs (16–18). The recently described CpG 7909 has been optimized to stimulate human plasmacytoid DCs (pDCs) and B cells in vitro and in vivo (18).

Clinical studies have shown that CpG 7909 is a potent inducer of human innate immune responses and exhibits a strong adjuvant effect when coadministered with vaccines eliciting B cell responses against hepatitis B virus (19, 20). In contrast, it remains to be determined whether CpG ODNs are efficient adjuvants for vaccine-induced human cytolytic T cell responses (7). Given the well-documented but still relatively weak antigen-specific CD8+ T cell responses observed recently in melanoma patients vaccinated with Melan-A_{26-35} peptide and IFA (21–23), we tested whether coadministration of CpG 7909 to the same vaccine would enhance T cell responses. We have therefore performed a phase I clinical trial to examine toxicity and immunogenicity of this approach. Our results show rapid and consistent T cell responses in vivo, highlighting the potential of CpG 7909 to enhance cellular immune responses in humans.

Results

Vaccination with CpG 7909, peptide, and IFA caused no major side effects. Eight HLA-A2+ patients with advanced melanoma disease received 4 monthly subcutaneous injections of low doses of CpG 7909, Melan-A analog peptide, and IFA. Vaccination with this novel

Nonstandard abbreviations used: CpG, deoxycytidyl-deoxyguanosin; IFA, incomplete Freund's adjuvant; Melan-A, melanoma antigen A; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; TLR, Toll-like receptor.

Conflict of interest: A.M. Krieg is an employee of Coley Pharmaceutical Group Inc. and owns stock in this company. The other authors have declared that no conflict of interest exists.

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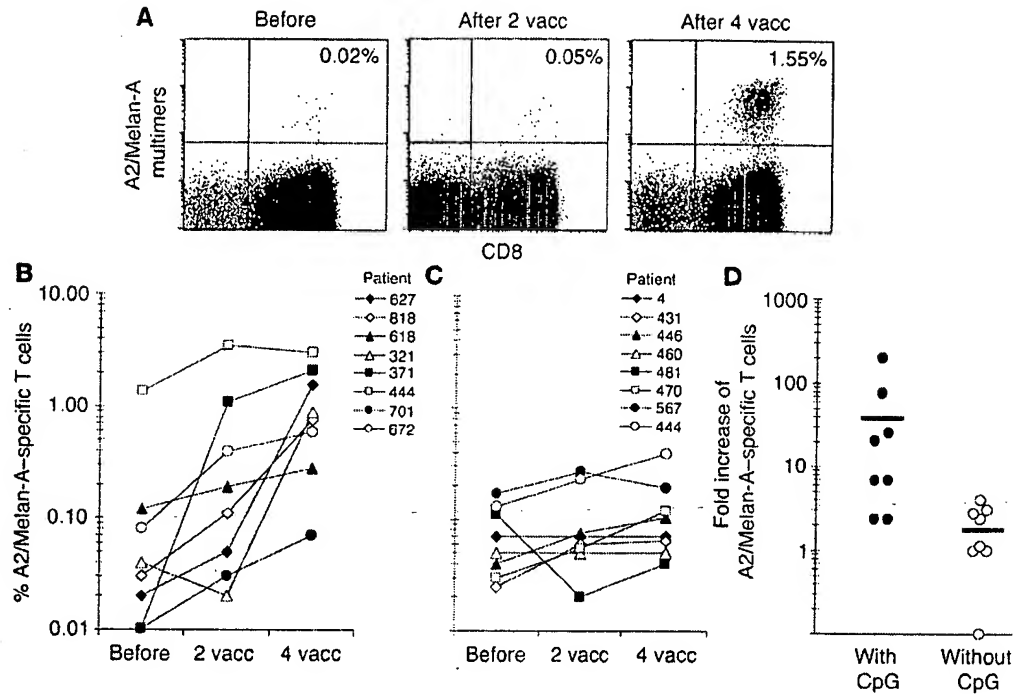

Figure 1

Rapid in vivo responses of Melan-A–specific T cells to vaccination (vacc) with low doses of CpG 7909, Melan-A peptide, and IFA. PBMCs were collected before, as well as 7–10 days after, vaccinations 2 and 4, and they were analyzed ex vivo by flow cytometry. (**A**) Dot plots from PBMCs of patient LAU 627, with percentage of Melan-A–specific cells among $CD8^+$ T cells. (**B**) After 2 and 4 vaccinations, 6/8 and 8/8 patients, respectively, had significantly increased percentages (i.e., greater than 2-fold) of Melan-A–specific T cells. (**C**) A control group of 8 patients was similarly treated with Melan-A peptide and IFA but without CpG (23). After 2 vaccinations, none of the patients had more than 2-fold increased percentages. After 4 vaccinations, 4/8 patients had more than 2-fold increased frequencies, but percentages of Melan-A–specific T cells remained significantly ($P < 0.01$) lower as compared to those of CpG-vaccinated patients. (**D**) Fold increase of Melan-A–specific T cells before or after 4 vaccinations in patients vaccinated with or without CpG. Horizontal lines indicate mean values.

3-component vaccine caused no major side effects. Minor systemic side effects were transient and included myalgia (4 patients), arthralgia and fatigue (3 patients), and nausea, malaise, and headache (2 patients). Interestingly, all 8 patients developed inflammatory signs at subcutaneous injection sites, with a peak of symptoms (induration, erythema, mild to moderate pain) around 2 weeks after injection. In response to recall vaccinations at distant sites in another limb, 4 patients showed reactivation of previous injection sites by redeveloping local inflammatory signs. Histological examination of a biopsy of one such distant reactivation site showed nonspecific inflammation with predominant perivascular lymphocyte infiltration (data not shown).

Rapid and consistent in vivo generation of Melan-A–specific $CD8^+$ T cells. PBMCs collected before and after vaccination were analyzed ex vivo by flow cytometry using fluorescent CD8-specific antibody and HLA-A2/Melan-A peptide multimers. After 4 vaccinations with CpG 7909, Melan-A peptide, and IFA, all 8 patients exhibited increased frequencies of Melan-A–specific $CD8^+$ T cells (0.07–3.00%), resulting in significantly ($P < 0.01$) higher percentages than before vaccination (Figure 1, A and B).

After 4 monthly vaccinations, the 8 patients exhibited a frequency of 1.15% ± 0.93% (mean ± SD) Melan-A–specific $CD8^+$ T cells. A control group of 8 melanoma patients was treated similarly but without CpG (Figure 1C), resulting in significantly ($P < 0.01$) lower T cell frequencies (0.13% ± 0.11%). Also, the response rate was far below 100%, since only 4 out of 8 patients generated ex vivo detectable T cell responses after 4 vaccinations. CpG-vaccinated patients reached a mean of 43-fold higher Melan-A–specific $CD8^+$ T cell frequencies than before vaccination, whereas patients vaccinated without CpG reached a mean of 1.9-fold higher frequencies than before vaccination (Figure 1D).

The majority of T cell responses developed rapidly: 6 out of 8 patients had increased frequencies of Melan-A–specific $CD8^+$ T cells after just 2 vaccinations (Figure 1B). This is again in sharp contrast to previous results. In the control group (Figure 1C), the lack of early responses (after 2 vaccinations) and the relatively low response rate are characteristic of all other studies with low-dose synthetic vaccines (21–25). Interestingly, patient LAU 444, who already had high levels of Melan-A–specific $CD8^+$ T cells before vaccination (resulting from previous immunotherapy and a natural response to melanoma [26]), was the only patient whose frequency peaked after just 2 vaccinations. In the other 7 patients, maximal frequencies were reached after 4 vaccinations. To our knowledge, these results demonstrate for the first time in humans that a synthetic peptide-based vaccine, when coadministered with adequate adjuvant, can rapidly elicit ex vivo detectable $CD8^+$ T cell responses.

Two of the 8 patients (LAU 371 and LAU 321) had previously been unsuccessfully vaccinated with Melan-A peptide mixed with the immunological adjuvants MPL and QS21 (Figure 2). During 4–5 years, the frequencies of Melan-A–specific T cells

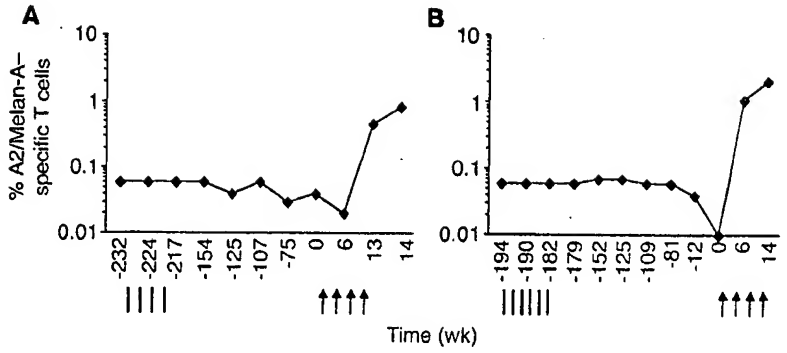

Figure 2
T cell responses to CpG vaccination (arrows) in patients that previously did not respond to vaccination with Melan-A peptide and immunological adjuvants MPL and QS21 (vertical lines). Percentages of multimer$^+$ T cells were determined ex vivo in PBMCs collected over an observation period of 4 to 5 years. (**A**) Patient LAU 321; (**B**) Patient LAU 371.

remained at stable low values (patient LAU 321, 0.05% ± 0.01%; patient LAU 371, 0.06% ± 0.02%), a frequent observation in immune nonresponders (27). Nevertheless, both patients responded rapidly and strongly to vaccination with CpG 7909, Melan-A peptide, and IFA (Figure 2).

T cell frequencies peaked 7–10 days after recall vaccination. Results in Figures 1 and 2 were obtained with blood samples collected 7–10 days following the monthly recall vaccinations (i.e., vaccinations 2 and 4). To determine the short-term kinetics of T cell responses, we analyzed additional blood samples collected immediately before and 14 days after recall vaccination with CpG 7909, Melan-A peptide, and IFA. The highest T cell levels were reached 7–10 days after recall vaccination (Figure 3). Maximal frequencies (as shown in Figures 1 and 2) were observed on day 7 in 4 patients (LAU 818, LAU 618, LAU 701, and LAU 672) and on day 10 in 3 patients (LAU 627, LAU 371, and LAU 444). Interestingly, frequencies of circulating Melan-A–specific T cells dropped to relatively low levels 14 days after recall vaccination (frequencies for patient LAU 321 were not evaluable, since blood samples from days 7 and 14 were not available). Thus, recall vaccination with CpG 7909, Melan-A peptide, and IFA led to increased T cell frequencies during the first 7–10 days, followed by a decline shortly afterwards, similarly to previous observations in mice (28–30).

Predominance of effector memory T cells expressing effector genes in vivo. It is well established that Melan-A–specific T cells in melanoma patients comprise naive and activated cells (31). To determine the differentiation state of vaccine-induced Melan-A–specific T cells, we first assessed CD45RA and CCR7 cell surface expression (32, 33) by Melan-A–specific T cells. Before vaccination, the majority were naive CCR7$^+$ CD45RA$^+$ T cells (Figure 4A). In contrast, after vaccination most Melan-A–specific T cells displayed an effector memory phenotype (CD45RA$^-$CCR7$^-$; Figure 4A and Table 1). After 4 vaccinations, effector memory cells accounted for 82% ± 13% of Melan-A multimer$^+$ T cells (mean ± SD of the 8 patients), and naive, central memory, and effector T cells were only found at low percentages. To assess effector gene expression directly, we sorted Melan-A–specific T cell subpopulations according to their expression of CD45RA and CCR7. As described previously (33), we sorted 5-cell aliquots and isolated mRNA, which was transcribed to cDNA then nonspecifically amplified and finally used for PCR with sequence-specific primers. All cells were positive for CD3 (Figure 4B). As expected, naive cells, which are not cytolytic and do not produce cytokines, did not contain detectable granzyme B, perforin, TNF-α, or NK receptor CD94 mRNA, and they only rarely gave an IFN-γ signal. After vaccination, granzyme B, perforin, and IFN-γ mRNA transcripts were found in significant fractions of 5-cell aliquots of effector memory cells. Finally, after 4 vaccinations, effector memory cells showed increased expression of granzyme B and perforin, and some fractions of 5-cell aliquots also expressed TNF-γ and NK receptor CD94.

Activated T cells with fine specificity to the natural tumor antigen. All vaccinations were done with the Melan-A analog peptide ELAGIGILTV, which contains the amino acid leucine in position 2 instead of the natural amino acid alanine. This substitution causes the analog peptide to bind more strongly and stably to HLA-A2, resulting in increased antigenicity and immunogenicity (31). To test the ability of T cells elicited by the vaccine to recognize A2/Melan-A antigen, we assessed the fine specificity of Melan-A–specific T cells in IFN-γ Elispot assays performed ex vivo. After vaccination, Melan-A–specific, IFN-γ–producing T cells from all 8 patients reached frequencies above the detection limit of 0.01% of CD8$^+$ T cells (Figure 5A and Table 2). The natural and analog peptides triggered comparable frequencies of Elispot-forming cells (0.12% ± 0.12% and 0.14% ± 0.10%, respectively). To assess the functional avidity, we tested the cytotoxic activity of A2/Melan-A–specific T cell clones derived from patients after vaccination. Cytotoxicity was peptide-specific and occurred at low peptide concentrations (Figure 5B), demonstrating high functional avidity to A2/Melan-A. The observed hierarchy of peptide recognition efficiency is characteristic for the majority of Melan-A–specific T cell clones and is in accordance with the hierarchy of binding strength of the 3 peptide variants to HLA-A*0201 (31). In agreement with these findings, the clones indeed killed the A2$^+$/Melan-A$^+$ melanoma cell line Me 275, but not the A2$^+$/Melan-A$^-$ melanoma cell line NA8 (Figure 5C). These data

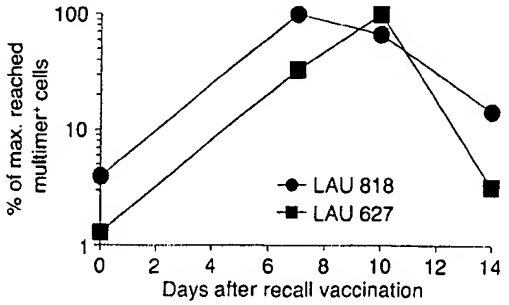

Figure 3
Short-term kinetics of Melan-A–specific T cells from 2 representative patients with maximal (max.) responses on day 7 (patient LAU 818) and on day 10 (patient LAU 627) after recall vaccination with CpG 7909, Melan-A peptide, and IFA. PBMCs were collected immediately before, as well as 7, 10, and 14 days after, recall vaccination. Percentages of multimer$^+$ T cells were determined ex vivo and calculated in percentages of maximally reached values (100%) per patient.

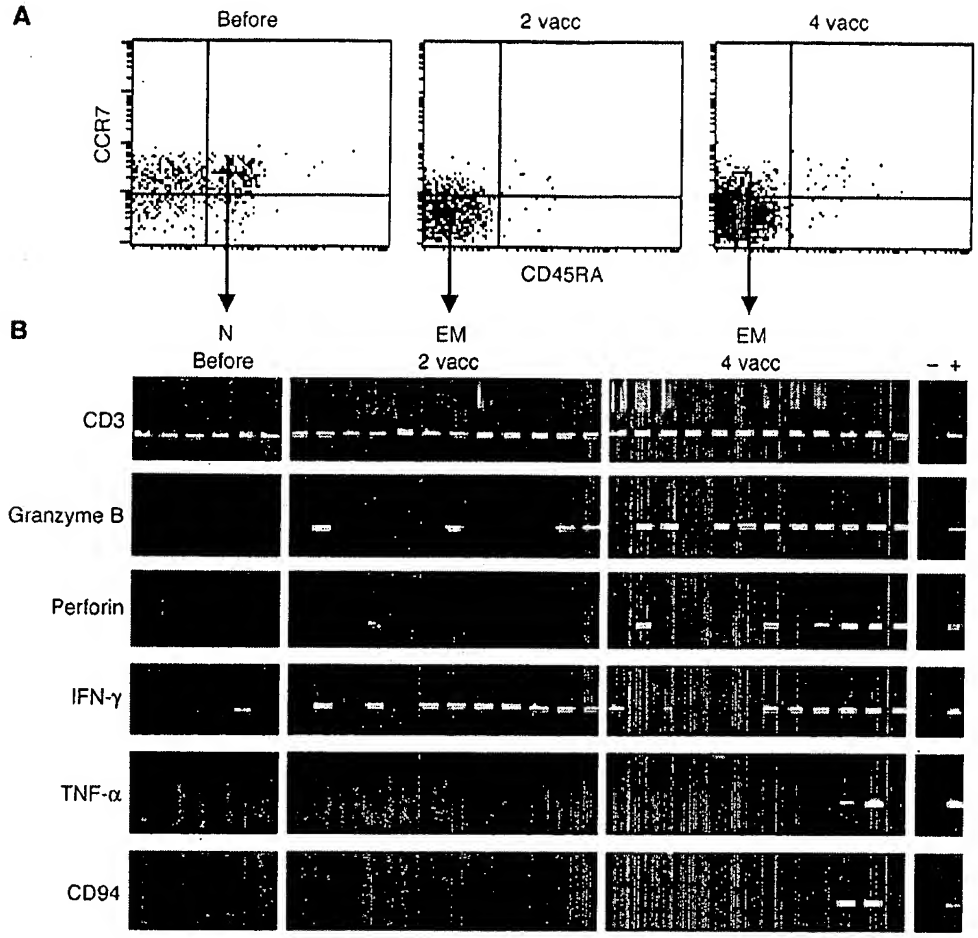

Figure 4
Expression of effector mediators by vaccine-activated Melan-A–specific T cells. Multiparameter cytometric analysis and sorting was performed with A2/Melan-A multimers and antibodies specific for CD8, CD45RA, and CCR7. (**A**) Expression of CD45RA and CCR7 is shown for A2/Melan-A multimer$^+$ CD8$^+$ gated cells. (**B**) RT-PCR gene expression analysis was performed (33) using primers specific for CD3, granzyme B, perforin, IFN-γ, TNF-α, and CD94, on sorted A2/Melan-A multimer$^+$ T cells which were RA$^+$CCR7$^+$ (naive cells) or RA$^-$CCR7$^+$ effector memory cells. Each band represents the RT-PCR product from RNA isolated from sorted 5-cell aliquots. Data in **A** and **B** (representative of 8 and 2 patients analyzed, respectively) are from PBMCs collected from patient LAU 371 before and after 2 and 4 vaccinations. +, positive control; –, negative control; N, naive T cells; EM, effector memory T cells.

demonstrate that T cells induced by a vaccine comprised of a synthetic Melan-A analog peptide specifically recognized the natural Melan-A antigen expressed by tumor cells.

Discussion

This study represents the first human trial of CpG ODN combined with a T cell peptide antigen. It demonstrates that CpG 7909 is an efficient adjuvant that promotes rapid antigen-specific CD8$^+$ T cell responses. As compared to vaccination without CpG, responses were 1 order of magnitude higher. Synthetic peptide plus adjuvant vaccines have been shown to induce only low frequencies of circulating antigen-specific T cells, despite administration of 8 or more injections (34, 35). Moreover, in the majority of cancer patient studies reported so far, T cell responses could not be detected ex vivo but rather only after 1 or more rounds of in vitro T cell stimulation and proliferation (21, 24, 36, 37). A small number of studies (using 0.5–1 mg peptide per vaccination plus adjuvants or cytokines) described ex vivo detectable T cell responses (22, 25), but T cell frequencies were still 10–100 times lower than what we observed in the present study. Only 1 study reported higher percentages of antigen-specific T cells following vaccinations with the particularly immunogenic gp100 T2M analog peptide and IFA. However, very high doses were used for vaccination: the cumulative peptide dose was 100-fold higher and the number of vaccinations 10-fold higher than in our study (38).

The HLA-A2/Melan-A antigenic system constitutes a well-defined model for studies of spontaneous and vaccine-induced CD8$^+$ T cell responses in humans (31, 37). Besides the gp100 analog peptide 2TM, Melan-A peptide is one of the rare cancer peptide antigens capable of inducing ex vivo detectable T cell responses in relatively large proportions of patients (22, 37). The availability of strong adjuvants such as CpG 7909 makes it possible to investigate the immunogenicity of weaker antigens, which make up the majority of known cancer epitopes.

Both CpG and peptide were given at low doses. Future studies will test whether increased doses of CpG 7909 and/or peptides can further enhance T cell activation. CpG 7909 can be administered to humans with generally acceptable tolerability at doses up to at least 20 mg per injection weekly for 6 months or longer (A. Krieg, unpublished observations), that is, at cumulative doses that are 250-fold higher than in our study. Very high CpG ODN doses (2.5 mg/kg) given daily for 20 days have recently been reported to destroy lymphoid tissue structures in mice (39). High-dose effects also include extramedullary hematopoeisis and a lethal systemic inflammatory response syndrome in mice (40). The potential toxicities of high-dose CpG 7909 administration in humans are likely to be largely different from those reported in mice, due to the much more restricted distribution of TLR9 expression in human as compared to mouse immune cells (5, 17). Our patients had no apparent liver toxicity, no enlargement of lymph nodes or spleen, and intact antigen-specific B

Table 1
Percentages of naive and non-naive Melan-A–specific T cells

	T_N	T_{CM}	T_{EM}	T_E
CD45RA	+	–	–	+
CCR7	+	+	–	–
LAU 627	0	4	93	2
LAU 818	0.7	7	89	3
LAU 618	0.1	8	91	0.8
LAU 321	5	30	60	5
LAU 371	0.7	15	84	0.6
LAU 444	1	1	93	5
LAU 701	2	5	62	31
LAU 672	5	7	86	3
Mean	2	10	82	6
SD	2	9	13	9

Percentages of naive (T_N; RA^+CCR7^+), central memory (T_{CM}; RA^-CCR7^+), effector memory (T_{EM}; RA^-CCR7^-), and effector (T_E; RA^+CCR7^-) T cells were determined among A2/Melan-A multimer$^+$ gated T cells from PBMCs collected 7–10 days after the fourth vaccination.

cell responses (data not shown). Two lymph node biopsies after CpG 7909 vaccination showed follicles with normal structure. Interestingly, 4 of 8 patients developed circulating anti-dsDNA antibodies as detected by ELISA, but there were no clinical signs for autoimmune disease, and systemic inflammatory markers in sera (IP-10, CRP) remained normal. We are currently investigating the possibility that the antibodies could be specific for CpG 7909.

Out of the 8 patients included in the study, 3 had no evidence for disease at study entry; during the study, 1 of them remained disease-free, 1 progressed, and 1 showed local tumor relapse. Among the 5 patients with measurable disease at study entry, 1 had stable disease, 2 progressed, and 2 showed local relapse. This phase I study was not designed to assess tumor response, and the follow-up time was short (mean of 7 ± 2 months after inclusion in the study). Comprehensive clinical results need to be obtained in phase II trials with more patients and a longer follow-up time.

In contrast to mouse TLR9, human TLR9 is only expressed by pDCs and B cells. Presumably, B cells are not involved in T cell activation, suggesting that pDCs were responsible for the observed T cell responses (41). One important question is whether our vaccine was capable of priming T cells, or whether the observed responses were due to reactivation and/or redistribution of previously primed cells. Remarkably, the levels of circulating naive Melan-A-specific T cells were abnormally low after CpG 7909 vaccination (undetectable in 4 patients, 0.03% ± 0.02% in the remaining 4 patients). For comparison, naive Melan-A-specific T cells are present at relatively high frequencies (0.07% ± 0.06%) in untreated healthy individuals and melanoma patients (42). Our observations that all patients responded, and that naive cell frequencies were low after vaccination, suggest that CpG 7909 vaccination was indeed capable of priming naive T cells. This conclusion is also supported by recent mouse experiments showing that CpG ODN-matured pDCs can prime $CD8^+$ T cells in vivo (41).

We also analyzed whether the prevaccination immune status differed between patients treated with and without CpG. However, we did not find significant differences with regard to prevaccination frequencies of naive cells or of each of the 3 other Melan-A-specific T cell populations defined by CD45RA/CCR7 expression. Data were interpretable from 3 patients of each group (data not shown). We have recently demonstrated that the percentage of preexisting CD28 negative Melan-A-specific T cells correlates with T cell responsiveness to vaccination with peptide and IFA in stage III–IV melanoma patients (26). Analyzing this aspect in the present study, we could not find a statistically significant difference between the 2 patient groups (mean prevaccination percentages ± SD of CD28 negative Melan-A-specific T cells were 28% ± 33% and 29% ± 27% in patients treated with and without CpG, respectively). Data were interpretable from 6 patients of each group (data not shown). Based on these findings, one may speculate that vaccination with CpG induces increased frequencies of circulating T cells independently of the patient's prevaccination immune status, whereas T cell responses to peptide and IFA vaccination (without CpG) depend significantly on endogenous (tumor-driven) immunogenicity (26).

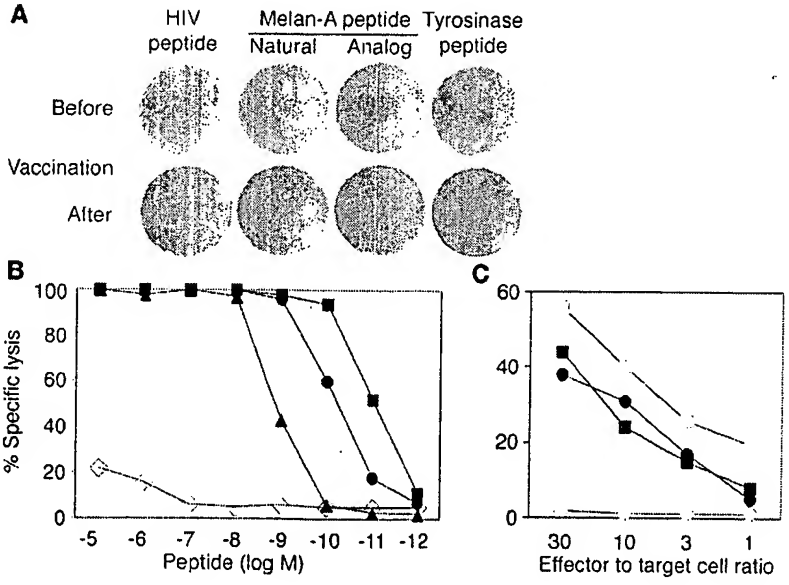

Figure 5
T cell receptor fine-specificity and tumor cell recognition. (**A**) PBMCs from patient LAU 371 were tested ex vivo in IFN-γ Elispot assays before and after vaccination with HIV, tyrosinase, and Melan-A natural and analog peptides. (**B**) Melan-A–specific T cells were sorted by flow cytometry and cloned, and cytotoxicity was tested against T2 cells in the presence of the following titrated HLA-A2 binding peptides: Melan-A analog (squares), Melan-A natural decamer (circles), Melan-A natural nonamer (triangles), and influenza matrix protein GILGFVFTL (diamonds). (**C**) Cytotoxicity against $HLA\text{-}A2^+$ melanoma cell lines Me 275 ($Melan\text{-}A^+$; filled symbols) and NA8 ($Melan\text{-}A^-$; open symbols) in the presence (squares) or absence (circles) of synthetic Melan-A analog peptide. Data shown were generated with clone 6 derived from patient LAU 371 and are representative for 12 of 22 clones generated from patients LAU 371 and LAU 444.

Table 2
Percentage of IFN-γ Elispot-forming cells

Patient	Melan-A peptide	
	Natural	Analog
LAU 627	0.08	0.08
LAU 818	0.05	0.06
LAU 618	0.07	0.13
LAU 321	0.02	≤0.01
LAU 371	0.11	0.12
LAU 444	0.40	0.32
LAU 701	0.02	0.02
LAU 672	0.19	0.23
Mean	0.12	0.14
SD	0.12	0.10

Percentages of Elispot-forming cells among $CD8^+$ T cells in PBMCs are shown for all 8 patients after vaccination. Background results with HIV polymerase peptide and without peptide were below the detection limit of 0.01% (not shown).

The pattern of specific T cell expansion and contraction following recall vaccination is reminiscent of acute T cell responses observed in mice (28–30). Interestingly, in patients vaccinated with Melan-A and IFA without CpG, such rapid changes of specific T cell frequencies were not observed (data not shown). In the majority of cancer vaccine studies reported so far, antigen-specific T cells were analyzed at only a single time point after each vaccination. With the introduction of highly efficient adjuvants such as CpG 7909, stronger, shorter-lasting T cell responses can be observed. Thus, antigen-specific T cells should be monitored at multiple time points after vaccination.

One possible explanation for the rapid decline during the second week after recall vaccination is T cell migration into lymphoid and tumor tissues (43). We were able to investigate metastases surgically removed from 3 patients after vaccination with CpG 7909, Melan-A, and IFA, and found that 5.03%, 0.12%, and 0.08% of $CD8^+$ T cells were specific for Melan-A (data not shown). Ongoing studies will address the question of whether vaccine-activated T cell clones are found in these lesions. We are also developing techniques to estimate the percentage of T cells that undergo apoptosis and/or migrate to other compartments (29, 44).

The vaccine-induced T cell populations consisted predominantly of effector memory T cells ($CD45RA^-$ $CCR7^-$). Central memory and effector cells were also induced, but to a much lesser extent. Most non-naive cells expressed IFN-γ mRNA. Effector memory cells expressed granzyme B mRNA, and some of them expressed perforin mRNA. While this was the case for the majority of 5-cell aliquots after 4 vaccinations, only a few cells were positive for granzyme B after only 2 vaccinations. Finally, TNF-α and CD94 were expressed by only a small fraction of cells. Through our ongoing studies, we aim to elucidate whether multiple recall vaccinations can induce effector functions in the majority of cells.

It is conceivable that the magnitude and durability of antigen-specific T cell responses induced by peptide-based vaccines could be further enhanced by the use of other types of CpG ODNs, such as the C type, which recently has been reported to induce higher levels of IFN-α secretion than the B type, which was used in this study (45–48). Indeed, IFN-α appears to improve the development of T cell memory (49, 50). Moreover, various other TLR ligands are becoming available for use in humans and are interesting candidates as adjuvants for vaccination and induction of protective immunity.

In conclusion, this study underscores the synergy of combined triggering of innate and specific immunity. The TLR9 ligand CpG 7909 is a potent stimulator of innate immune cells, and its coadministration with antigen induces high numbers of antigen-specific $CD8^+$ T cells. Our findings suggest that further approaches to T cell–directed immunotherapy should take advantage of such a synergy.

Methods

Patients, eligibility criteria, and study protocol. $HLA\text{-}A2^+$ patients with histologically proven metastatic melanoma of the skin expressing Melan-A/MART-1 (determined by RT-PCR or immunohistochemistry) were included after informed consent was obtained in this phase I prospective trial of the Ludwig Institute for Cancer Research (LICR). Study protocols were approved by the LICR protocol review committee, as well as by the medical and ethical committees of the University Hospital (Lausanne, Switzerland). Inclusion criteria were as follows: Karnofsky performance status of at least 70%, normal complete blood count and kidney-liver function, and no concomitant anti-tumor therapy or immunosuppressive drugs. Exclusion criteria were pregnancy, seropositivity for HIV-1 Ab or hepatitis B surface antigen, brain metastasis, uncontrolled bleeding, clinically significant autoimmune disease, or New York Heart Association class III–IV heart disease. Study end points were toxicity and $CD8^+$ T cell response. All patients were evaluated for immune response and treatment toxicity. Patients received 4 vaccinations subcutaneously in monthly intervals. The low-dose vaccines were composed of 500 μg CpG 7909, 100 μg Melan-A analog peptide, and 300 μl IFA (Montanide ISA-51; Seppic) all mixed together and prepared in a syringe as a stable emulsion. Phosphorothioate backbone CpG 7909 (TCGTCGTTTTGTCGTTTTGTCGTT) was produced by Coley Pharmaceutical Group under good manufacturing practice conditions, and had no detectable endotoxin by limulus amebocyte lysate assay. The Melan-A analog $peptide_{26\text{-}35}$ ELAGIGILTV was synthesized by NeoMPS Inc. and formulated (330 μg/ml in PBS/30% DMSO) by the Biological Production Facility, LICR Melbourne. The IFA (Montanide ISA-51; Seppic) contained mineral oil (Drakeol) and anhydro mannitol octadecanoate. Control patients were treated similarly with 4 monthly vaccinations as described previously (23), except the vaccines did not contain CpG ODN. As in most phase I studies, patients were not randomized, but first assigned to the control group and subsequently to the CpG group. Patient LAU 444 was first included in the control group, and 2 years later in the CpG group.

Blood cells, HLA-A2/peptide multimers, flow cytometry, and IFN-γ Elispot assays. Ficoll-Paque centrifuged PBMCs (1×10^7–2×10^7) were cryopreserved in RPMI 1640, 40% FCS, and 10% DMSO. Phycoerythrin-labeled HLA-A*0201/peptide multimers (originally called tetramers) were prepared as described previously (42, 51) with Melan-A analog $peptide_{26\text{-}35}$ ELAGIGILTV. Anti-CD8, anti-CD28, and allophycocyanin-conjugated goat anti-rat IgG were purchased from BD Biosciences and anti-CD45RA was from Immunotech. Anti-CCR7 rat IgG2a mAb 3D12 was provided by Martin Lipp (Max Delbrueck Center for Molecular Medicine, Berlin, Germany). Five color stains were done with HLA-A2/peptide multimers, FITC-conjugated anti-CD28, PE-Texas Red–conjugated anti-CD45RA, allophycocyanin-Cy7–conjugated anti-CD8 reagents, and anti-

CCR7 mAb followed by allophycocyanin-conjugated goat anti-rat IgG antibody. Briefly, $CD8^+$ T cells were enriched using a MiniMACS device (Miltenyi Biotec) resulting in more than 90% $CD3^+CD8^+$ cells. Cells (10^6) were incubated with multimers (1 μg/ml, 60 minutes, room temperature) and then with antibodies (30 minutes, 4°C). We acquired 5×10^5 $CD8^+$ T cells per sample with a FACSVantage machine, and data were analyzed with CellQuest software (BD Biosciences). IFN-γ Elispot assays were performed using IFN-γ–specific antibodies (Diaclone, Biotest). Briefly, plates were coated overnight with antibody to human IFN-γ and washed 6 times. We added 1.66×10^5 PBMCs/well in 200 μl Iscove medium (Gibco; Invitrogen Corp.) supplemented with 8% human serum and 10 μg/ml peptide and incubated it for 16 hours at 37°C. Assays were performed in 6 replicates, without peptide or with peptides derived from Melan-A (natural EAAGIGILTV, analog ELAGIGILTV); tyrosinase YMDGTMSQV, and HIV-1 polymerase ILKEPVHGV. Cells were removed, and plates were developed with a second (biotinylated) antibody to human IFN-γ and streptavidin-alkaline phosphatase (Diaclone, Biotest). The spots were revealed with BCIP/NBT substrate (Sigma Tablets; Sigma-Aldrich) and counted with an automatic reader (Bioreader 2000; BioSys GmbH). The percentage of $CD3^+CD8^-$ PBMCs was determined by flow cytometry on the same batch of cryopreserved cells. Results of both multimer$^+$ T cells and Elispot-forming T cells were calculated as a percentage of $CD8^+$ T cells. For each patient and assay system, pre- and post-vaccination samples were thawed and tested in the same experiment.

Quality control of laboratory immune monitoring. Standardization of multimer and IFN-γ Elispot assays was done with 180 unselected blood samples from healthy donors and patients (27). For both multimer and IFN-γ Elispot assays, the detection limit was 100 cells in 10^6 $CD8^+$ T cells (0.01%) (42). Repeated analyses showed 15% ± 16% (mean ± SD) variation in multimer$^+$ cells (37). Good reproducibility was also found for the Elispot assay (variation of 30% ± 21%). In addition, longitudinal intraindividual result variability was assessed by testing multiple blood samples from individuals without a T cell response against Melan-A. Variation coefficients (SD in percent of mean multimer$^+$ values) were 20% ± 27%. For IFN-γ Elispot assays, negative controls with all PBMC samples had a mean of less than 0.003% spots among $CD8^+$ T cells, confirming that the background was far below the detection limit of 0.01%.

Cell sorting, cDNA amplification, and 5-cell RT-PCR. $CD8^+$ T cells were enriched with a MiniMACS device and stained with multimers and antibodies as described above. Five-cell aliquots were sorted directly with a FACSVantage SE machine into wells of 96-V bottom plates. cDNA preparation, cDNA amplification, and PCR were done using primers for CD3, granzyme B, perforin, IFN-γ, TNF-γ, and CD94 as described (33). Either water or Daudi B cell line extract was used as a negative PCR control (–); 10^3 PBMCs from a healthy individual were used as a positive control (+).

T cell cloning and cytotoxicity assay. Multimer$^+$ $CD8^+$ T cells were sorted by flow cytometry, cloned by limiting dilution, and expanded with phytohemagglutinin (PHA) and allogenic feeder cells in medium containing 150 U/ml human recombinant IL-2 (hrIL-2) and 10 ng/ml hrIL-7. Subsequently, they were periodically (every 3–4 weeks) restimulated with PHA, irradiated feeder cells, and hrIL-2. Lytic activity and antigen recognition were assessed functionally in 4-hour ^{51}Cr release assays (42). Target cells were T2 cells ($A2^+$/Melan-A^-) and the melanoma cell lines Me 275 ($A2^+$/Melan-A^+) and NA8 ($A2^+$/Melan-A^-) (31). The percentage of specific lysis was calculated as follows: 100 × ([experimental – spontaneous release]/[total – spontaneous release]).

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remission was obtained in 31 cases (82%), partial response in 2 cases (5%), progression in 2 (5%), and 3 patients were not evaluated. Nineteen patients relapsed (50%), ten (26%) with systemic relapse, four (13%) local and five (13%) locoregional failure. At the moment of the analysis the median follow-up is 64 months (limits 12-233), 14 patients are alive and disease-free (37%), 5 patients are alive in relapse (13%) and 19 patients died, 16 (42%) secondary to tumor progression and 3 (8%) by other causes. Median survival of 54 months and ten year survival of 38%. Median survival in patients treated before 1990 was 47 months; in patients treated after that date the median survival has not been arised (p=0.49). Median survival in patients treated only with RT was of 47 months and in patients in which CT was added the survival was of 53 months (p=0.19). CONCLUSIONS: Undifferentiated nasopharyngeal carcinoma is an uncommon tumor. Median age of the patients is lower than age of diagnosis of head and neck cancer. The disease appears at advanced stages. RT is the main therapeutic modality and the overall response rate must be near 70%. Combination treatment (RT-CT) improves the results and is considered today the standard treatment.

MELANOMA AND SARCOMA

5770 Incidence, prevalence, phenotype and biologic spectrum of gastrointestinal stromal cell tumors (GIST) - A population-based study of 600 cases

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The recent refinement of diagnostic criteria for GIST and particularly the ability to detect the KIT receptor immunohistochemically with CD117 have now made it possible to determine the true incidence and prevalence of GIST. With the development of the remarkably effective new treatment modality Glivec (imatinib, formerly STI571, Novartis), a KIT receptor tyrosine kinase inhibitor, further definition of diagnostic criteria and prognostic markers has become extremely important. Most previous large studies of GIST are probably biased regarding its spectrum of biologic behavior, since they have been based primarily on cases that have been diagnosed and/or treated at a referral center. Our study aims to analyze a well-defined population from southwestern Sweden (population approximately 1.5 million) where all histopathologic diagnoses have been made in four pathology laboratories. All potential cases of GIST diagnosed between 1983 and 2000 have been retrieved and reviewed, resulting in a total of 600 GIST fulfilling diagnostic criteria which included CD117 immunoreactivity. Histologic and immunophenotypic features have been analyzed as well as details of clinical presentation, behavior, treatment, and prognosis. In addition, material has been obtained for a tissue array bank and for KIT mutation analysis. All data is currently being subjected to multivariate statistical analysis. Our results indicate that: 1) symptomatic/clinically detected GIST occur with an incidence of approximately 20 cases/million inhabitants per year; 2) GIST is a rather common incidental finding at surgery, endoscopy, or autopsy; 3) histologically GIST has been underrecognized and its malignant potential has been underestimated; 4) the histologic spectrum of GIST is wider than previously recognized; and 5) a small subgroup of GIST lacking CD117 immunoreactivity can be defined.

5780 Fotemustine (F) *versus* Dacarbazine (DTIC) as first line treatment in disseminated malignant melanoma (MM) with or without brain metastases (BM): A randomized phase III trial

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This phase III study compared F (100mg/m² iv weekly, 3 weeks) and DTIC (250 mg/m²/d iv, 5 days every 3 weeks, 2 cycles) in terms of objective response rate (OR), safety profile, quality of life (QoL) using QLQ-C30 scores, and overall survival (OS). Non-progressive patients (pts) received a maintenance therapy every 3 weeks (F 100 mg/m² or DTIC 250 mg/m²/dx5). From 02.98 to 09.00, 229 pts were randomized to receive F (n=112) or DTIC (n=117). In the Full Analysis Set, the OR was 15.5% (F) *versus* 7.2% (DTIC) (p=0.053). In pts without BM at inclusion (n=182), the median time of BM occurence was 22.7 (F) *vs* 7.2 months (DTIC) (p=0.059). The main toxicity was grade 3-4 neutropenia (51% of pts in F arm *vs* 5% in DTIC arm) and thrombocytopenia (43% in F arm *vs* 6% in DTIC arm) occuring mainly during induction. Severe non-hematological toxicity was infrequent, including pain (4 pts in F *vs* 17 in DTIC) and nausea-vomiting (3 pts in F *vs* 4 in DTIC). No toxic death occured. No significant difference was noted for QoL results between arms (n=156). Subgroup analyses showed a general trend for deteriorating QoL in progressive pts and stable or improving QoL for others. At time of analysis, the median OS was 7.4 (F) *vs* 5.8 months (DTIC) (p=0.073). A positive trend in favor of F in terms of OR, OS, and time to BM occurence was seen in first line treatment of MM. Both treatments had acceptable safety profiles and no difference was noted in QoL scores.

5790 Phase I/II study with CpG 7909 as adjuvant to vaccination with MAGE-3 protein in patients with MAGE-3 positive tumors

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Vaccination is a new rapidly expanding modality in antitumor immunotherapy. To improve effectiveness of anticancer vaccines powerful immunological adjuvants are needed. Recently, specific synthetic oligodeoxynucleotide sequences demonstrated immunostimulatory effects. The dinucleotide CpG 7909 is a novel promising adjuvant for induction of both humoral and cell-mediated immune responses. Furthermore, MAGE-3 encoded tumor-specific antigens are established targets for anticancer vaccine therapy. In a ongoing phase I/II study patients with metastatic MAGE-3 positive melanoma were vaccinated with CpG 7909 and MAGE-3 protein to evaluate safety, tolerability, immunological reactiveness and antitumor activity. Eligibility was according to standard criteria. Patients known with autoimmune diseases were excluded. Patients were vaccinated intramuscularly on days 1, 22, 43 and 64. Patients without progression received further vaccinations on days 92, 113 and 134. We studied 2 different dose levels of CpG 7909 (500 and 1000 microgram). The MAGE-3 protein dose was fixed at 300 microgram. Presently 13 patients have been enrolled, 8 in cohort 1 and 5 in cohort 2. Eleven patients received at least 2 vaccinations, one 2, one 3, two 4, one 6, four 7, one 12 and one 15 vaccinations, respectively. Two patients in cohort 1 were not evaluable due to rapid progressive disease (1) and intercurrent neurologic paraneoplastic syndrome (1). Treatment was well tolerated with transient grade I and II toxicities consisting of fever, fatigue, myalgia, headache, nausea/vomiting, tenderness and skin reaction at the injection site with no apparent CpG 7909 dose-related differences. One patient showed stable disease (12+ months) and 1 patient developed a partial response (9+ months). Preliminary immunological analysis showed an increase (10-150X) in anti-MAGE-3 antibody titers with a

stronger reaction in the 1000 microgram CpG dose cohort. In conclusion, vaccination with the immunological adjuvant CpG 7909 up to a dose of 1000 microgram in combination with the MAGE-3 protein is well tolerated and safe. The preliminary results of this study support further research.

580O Phase I trial of dexosome vaccine for patients with advanced melanoma: Final results

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Dexosomes (Dendritic cell-derived EXosomes), 60-90nm vesicles released by immature monocyte-derived dendritic cells (MD-DC), can be efficiently loaded with tumor peptides to induce potent T cell stimulation. A Phase I safety and feasibility study of DEX vaccine therapy was conducted in 15 patients with advanced melanoma. Eligibility requirements: Stage III/IV melanoma, HLA A1/B35 and DP04 haplotype, tumor Mage-3 expression and + recall skin testing. The DEX were isolated from DC culture supernatant from a single leukapheresis (except in 2 pt). Tumor peptides (Mage-3.A1/Mage-3.DP04 were loaded onto the DC during culture (6 pts); or loaded directly onto the purified dexosomes (9 pts). Escalating doses of cryopreserved DEX were administered by 4 weekly id/sc injections, then every 3 weeks in patients who achieved stable disease or a tumor regression.. Fifteen patients have entered the study and 13 have completed vaccine therapy as of June 1st, 2002. Patient characteristics: median age 59 (range 29-76); M:F, 8:7; Stage; III 5 pts, IV 10 pts; 53% had prior chemotherapy; 27% had prior immunotherapy; 4 patients had an elevated LDH at entry. All patients had progressive disease before treatment, sites of metastatic disease included skin, lung, nodes and liver. Results: The vaccine therapy was well tolerated, without evidence of serious toxicity. Among the 13 patients who have completed treatment, one patient with Stage III disease achieved stable disease following the first 4 injections, received 8 additional vaccine injections with continued stable disease 15+ months. One partial response on lymph nodes and one "mixed" response have been observed at the highest level. Immunomonitoring results will be presented including lymphocytes microcultures screened by MAGE.3-A1/B35 tetramers allowing isolation of specific CTL clones in stable or responding patients. This phase I trial demonstrate that administration of DEX is feasible and safe with encouraging clinical and immunological responses to support a phase II study in melanoma. Clinical and immune responses of all 15 patients will be presented and updated.

581P Prognostic factors in radically resected malignant melanoma

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Introduction: The c-kit gene encodes a transmembrane tyrosine kinase receptor and plays an important role in tumor growth, invasion and metastatic spread of malignant melanoma. Expression of c-kit was investigated in preclinical studies. No large clinical study has been done thus far. Patients and Methods: Primary tumor specimens of 189 radically resected patients with stage I and II malignant melanoma were examined for the presence of c-kit expression. Formalin-fixed, paraffin embedded, tissues were stained with the polyclonal rabbit anti-human c-kit antibody (Dako®). The univariate and multivariate analysis of c-kit expression and other clinical (disease free survival) or pathological features (Breslow, Clark, microscopic ulceration) have been performed. Results: In the population of 189 early stages melanoma patients occurred 37 recurrences. Among investigated samples were 121 (64%) negative for c-kit expression, 57 (30%) samples were positive in less than 50% of cells and 11 (6%) samples were positive in more than 50% of cells. In univariate analyses, high Breslow, Clark and the presence of microscopic ulceration were significantly associated with shorter disease free survival (respectively: $p<0.000001$; $p<0.0007$; $p<0.0001$). c-kit overexpression almost reached statistical significance ($p<0.0594$) as a negative prognostic factor. In multivariate analyses the combination of Breslow, ulceration and c-kit appeared useful in the assessment of patient prognosis (respectively: $p<0.0002$, $p<0.0062$, $p<0.0142$). When all parameters were correlated to each other the only strong positive correlation was found between Breslow and Clark ($r=0,61$; $p = 0.0001$). Conclusion: Breslow, Clark and microscopic ulceration were only statistically significant prognostic factors. However, c-kit expression almost reached the significance. The combination of Breslow, ulceration and c-kit assessment appeared to be ideal combination in determination of prognosis. The small number of events in the study group might influence these results. Acknowledgment: This research was supported by the grants of the League Against Cancer, Terry Fox Foundation and CEZ a.s.. Thanks to Dr. Julis for the c-kit expression determination.

582P Accuracy of sentinel lymph node and positron emission tomography scanning in the detection of micro-metastases of malignant melanoma

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Purpose: The pathologic status of the sentinel lymph node (SN) is a powerful indicator of prognosis in patients with melanoma. 18-fluoro-2-deoxy-D-glucose positron emission tomography (18FDG-PET) scanning is a non invasive imaging technique that can detect clinically silent metastases. The aim of this study is to compare the use of 18FDG-PET and sentinel lymph node biopsy (SNB) in detecting metastatic disease in the regional draining nodes of cutaneous malignant melanoma. Patients and methods: Twenty two consecutive patients (14 women and 8 men; mean age 48 years) with primary cutaneous melanoma = 1mm or <1mm and Clark level IV-V, ulceration or lymphovascular invasion were recruited between March 1998 and January 2002. Mean Breslow thickness was 1.9 mm (range 0.75- 7 mm). The sites of primary melanoma were 11 on the extremities, 6 on the trunk and 5 on head and neck. Ulceration was present in 2 lesions. Distant metastases were excluded by clinical exploration, biochemical profile and chest x-ray. PET scanning was followed by a preoperative lymphoscintigraphy with 99mTc sulphur colloid to identify SNs. All patients with positive SNB underwent therapeutic lymph node dissection (LND). Results: The SNs were identified in 100% of the patients. A total of 32 SNs were detected and removed. In four patients (18%), histopathology revealed lymph node metastases (4 lymph nodes). Regional LND demonstrated one more lymph node metastasis in one patient. In none of these 4 patients PET scans identified metastatic disease in the SN or draining basin. Two patients had positive PET scans suspicious of metastatic disease, both of them with a negative SNB. No patient has developed recurrent disease (mean follow-up, 7 mounths). Conclusions: This study demonstrates the limitations of 18FDG-PET in detecting lymph node micrometastases in patients with primary malignant melanoma compared with SNB.

583P Low prevalence of microsatellite instability in malignant melanoma

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Background: Malignant melanoma (MM) is thought to arise by sequential accumulation of genetic alterations in normal melanocytes. To clearly assess the role of genetic instability in MM, we have compared allelic alterations (detected as microsatellite instability; MSI) between primary tumors and synchronous or asynchronous metastases obtained from the same MM patients. Methods: Paraffin-embedded tissue microdissections from 56 MM patients were screened for MSI by PCR-based microsatellite analysis. MSI was studied at five loci containing single- or dinucleotide repeat sequences and mapping to different chromosomal locations. Tumors were classified as MSI+ when at least two markers displayed mutant alleles in tumor DNA compared to corresponding normal tissue DNA. Results: Presence of MSI, which may reflect a defect in genes involved in DNA replication fidelity, was observed in 5 (9%) out of 56 primary MM tumors. When the same series of patients has been analyzed for the presence of MSI in the corresponding metastases, we found a higher incidence (7/42, 17%) of

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CpG Motifs Are Efficient Adjuvants for DNA Cancer Vaccines

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DNA vaccines can induce impressive specific cellular immune response (IR) when taking advantage of their recognition as pathogen-associated molecular patterns (PAMP) through Toll-like receptors (TLR) expressed on/in cells of the innate immune system. Among the many types of PAMP, immunostimulatory DNA, so-called CpG motifs, was shown to interact specifically with TLR9, which is expressed in plasmacytoid dendritic cells (pDC), a key regulatory cell for the activation of innate and adaptive IR. We now report that CpG motifs, when introduced into the backbone, are a useful adjuvant for plasmid-based DNA (pDNA) vaccines to induce melanoma antigen-specific protective T cell responses in the Cloudman M3/DBA/2 model. The CpG-enriched pDNA vaccine induced protection against subsequent challenge with melanoma cells at significantly higher levels than its parental unmodified vector. Preferential induction of an antigen-specific, protective T cell response could be demonstrated by (I) induction of antigen-dependent tumor cell protection, (II) complete loss of protection by *in vivo* $CD4^+/CD8^+$ T cell- but not NK cell-depletion, and (III) the detection of antigen-specific T cell responses but not of relevant NK cell activity *in vitro*. These results demonstrate that employing PAMP in pDNA vaccines improves the induction of protective, antigen-specific, T cell-mediated IR.

Key words: CpG motifs/CTL/melanoma/pDNA/tumor immunity
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Most tumors express antigens (TAA) in the context of major histocompatibility molecules (MHC) that can be recognized by the adaptive arm of the immune system. There is now a considerable body of data from animal models and human cells *in vitro* that indicates that T cells are the major effector cell population for the immunological control of TAA-expressing tumor cells (reviewed in Rosenberg, 2001; Finn, 2003). Nonetheless, tumors frequently escape spontaneous recognition and subsequent destruction by the immune system. Failure to generate optimal tumor antigen-specific T cell responses may be one reason for this phenomenon (Finn, 2003). In the case of melanoma, several groups of TAA have been identified so far, including the so-called melanocytic differentiation antigens (MDA) such as Pmel17/gp100 (Cox *et al*, 1994; Kawakami *et al*, 1995; Wagner *et al*, 1995). The identification of these antigens now allows the development of active immunization strategies to either induce or enhance antigen-specific T cell responses. The optimal immunization strategy for inducing such cellular immune responses (IR) in humans remains undefined and animal models of melanoma may be a valuable tool to test various vaccine formulations (Finn, 2003).

Plasmid-based DNA (pDNA) vaccines have not just been able to induce antibody but also cellular IR in laboratory animals and in humans (reviewed in Donnelly *et al*, 1997). In humans, this has been demonstrated particularly for pathogen-derived antigens with the induction of antigen-specific antibody as well as T cell responses (Wang *et al*, 1998; Tacket *et al*, 1999; Roy *et al*, 2000; Wang *et al*, 2001) that in a prime-boost strategy with modified vaccinia virus Ankara may also translate into T cell-mediated protective immunity (McConkey *et al*, 2003). In melanoma patients, lymph node infusion of pDNA vaccines led to the induction of antigen-specific T cells (Tagawa *et al*, 2003) and intratumoral injection led to regression of tumors at distant sites in a minor proportion of patients (Stopeck *et al*, 1997). A recurring observation made in all these clinical trials is that although it appears possible to induce cellular IR with pDNA vaccines in humans, relatively high doses of DNA are required to elicit detectable but still weak IR (Calarota *et al*, 1998; Wang *et al*, 1998; Tagawa *et al*, 2003). Therefore, strategies to optimize the immunogenicity of pDNA vaccines are clearly needed. One strategy to modulate significantly the immunological efficacy of a vaccine is the use of adjuvants, particularly if the immunogenicity of a given antigen is limited, as in the case of TAA (Cohen *et al*, 1998).

Bacterial extracts are known as potent activators of both innate and adaptive IR and are recognized by the immune

Abbreviations: ADCC, antibody depending cytotoxicity; AdV, adenovirus; IFN, interferon; IL, interleukin; IR, immune response; LAK, lymphokine activated killer; MDA, melanoma differentiation antigen; ODN, oligodeoxynucleotide(s); PAMP, pathogen-associated molecular patterns; pDC, plasmacytoid dendritic cell(s); pDNA, plasmid DNA; PFA, paraformaldehyde; TAA, tumor associated antigens; TLR, Toll-like receptors

system via "pattern-recognition receptors" that are expressed on distinct cells of the innate immune system. The so-far best-characterized member of "pattern-recognition receptors" is the Toll-like receptor (TLR) family. TLR9 is expressed in the endosomal compartment exclusively of plasmacytoid dendritic cells (pDC) and B cells in humans and, in addition, on myeloid DC and monocytes/macrophages in mice (Kadowaki *et al*, 2001; Krug *et al*, 2001; Ahmad-Nejad *et al*, 2002; Hornung *et al*, 2002). It is now known that the molecular structure recognized in bacterial extracts by TLR9 consists of unmethylated CpG dinucleotides in certain base contexts, the so-called CpG motifs (Krieg *et al*, 1995; Krieg, 2003). When present in synthetic oligonucleotides (ODN), CpG motifs are able to enter the lysosomal compartment of pDC within minutes of exposure, where these motifs interact specifically with TLR9. Downstream signaling involves the adapter molecule MyD88 and ultimately leads to activation of pDC and maturation into professional antigen-presenting cells (Krug *et al*, 2001). These express co-stimulatory molecules, the chemokine receptor CCR7, and secrete Th1-promoting chemokines and cytokines such as IP-10 and type I interferon (IFN) (reviewed in Krieg, 2002). Within hours, secondary effects such as NK cell activation, enhanced expression of Fc receptors on polymorphonuclear leukocytes, and an increase in antibody-dependent cellular cytotoxicity (ADCC) are induced. This stimulation of the innate immune system with activation/maturation of pDC and antigen presentation in a Th1-like cytokine milieu can induce primary CD8 T cell responses even in the absence of CD4 T cell help (Wild *et al*, 1999; Sparwasser *et al*, 2000).

We have previously examined an animal model of melanoma to define the prerequisites necessary for and the mechanisms involved in the induction of protective immunity by an antigen-specific pDNA vaccine (Wagner *et al*, 2000). The goal of this study was to determine whether CpG motifs can act as an adjuvant to the induction of antigen-specific cytotoxic T lymphocyte (CTL) responses against a weakly immunogenic TAA delivered as pDNA vaccine and if this translates into enhanced protective immunity against melanoma cells. The results obtained suggest that the introduction of CpG motifs into backbone sequences of the vector represents a promising strategy to enhance the immunogenicity of pDNA-based cancer vaccines.

Results

pUK21-A2 vector and its CpG-enriched variant pMCG16 exhibit comparable transgene expression levels To check whether the modification of the pDNA vector for its content of CpG motifs may have altered expression levels of the transgene, we transfected COS-7 cells with either pUK21-A2/EGFP or pMCG16/EGFP and, 48 h later, compared their EGFP-fluorescence levels as a function of transgene expression (EGFP, enhanced green fluorescent protein). FACS analysis revealed that both vectors yielded similar transfection efficiencies with regard to the frequency of transfected cells as well as transgene expression levels (Fig 1). These results confirm that the enrichment of CpG motifs into vector pUK21-A2 had no direct effect on transgene expression.

Protective anti-tumor activity of pDNA vaccines is significantly augmented by CpG motifs The generation of Cloudman S91 M3-derived melanoma sublines M3-7 (Pmel17/gp100$^+$) and M3-1 (Pmel17/gp100$^-$) has been described earlier (Wagner *et al*, 2000). S.c. inoculation of 5×10^5 cells of both sublines into syngeneic DBA/2 mice regularly results in the appearance of exponentially growing tumors at the injection site without subsequent formation of macroscopically detectable visceral metastasis.

To compare pDNA vectors pUK21-A2/mPmel17 and pMCG16/mPmel17 for their ability to induce anti-tumor immunity, mice received two injections of the respective constructs and were subsequently challenged with Pmel17/gp100$^+$ M3-7 cells. Twelve of 26 animals (46.2%) immu-

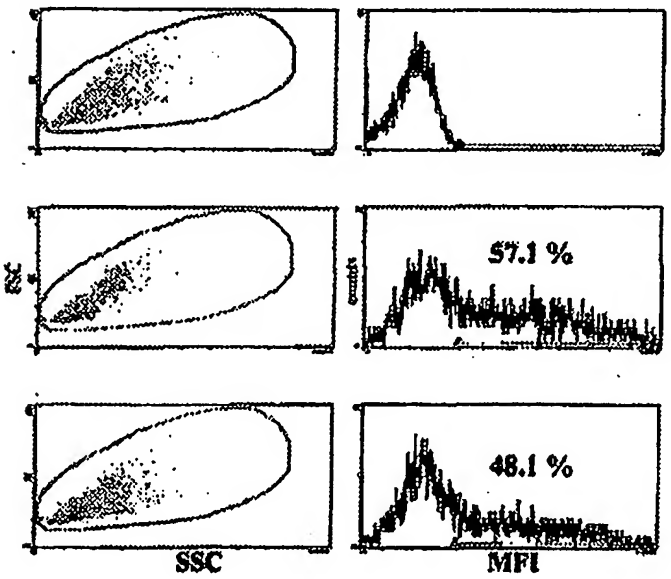

Figure 1
Intracellular expression level of enhanced green fluorescent protein (EGFP) in COS cells after transfection with CpG-enriched pMCG16 and non-CpG-enriched pUK21-A2 pDNA. Fifty percent confluent COS cells were transfected with either pUK21-A2/EGFP or pMCG16/EGFP. Their EGFP fluorescence was compared 48 h later by FACS analysis. Expression levels of the EGFP transgene from the parental pUK21-A2 vector (middle) are comparable with those derived from the CpG-enriched pMCG16 vector (bottom). Mock-transfected COS cells served as negative control (top).

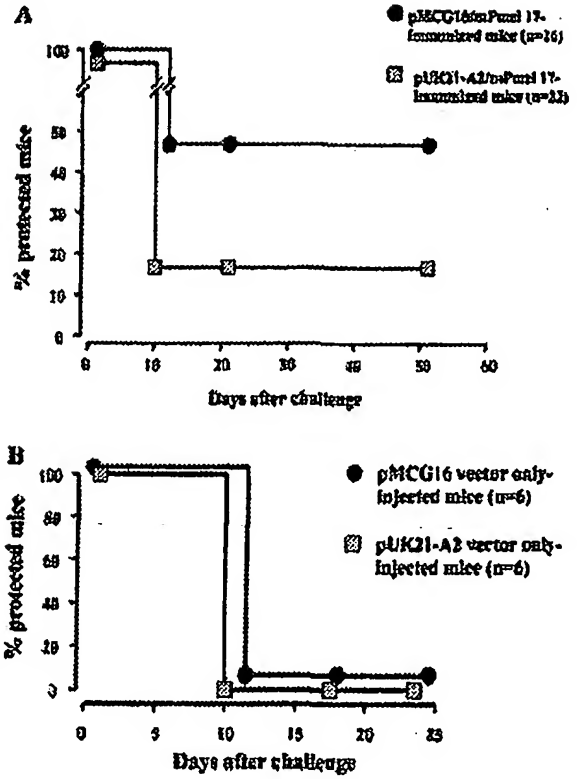

Figure 2
Increased protection against melanoma cell challenge obtained after genetic vaccination with a CpG-enriched plasmid-based DNA (pDNA) vector construct. Mice were injected interdermally into one side of the back with 100 μg of either pUK21-A2/mPmel17 or pMCG16/mPmel17 pDNA constructs, twice in a 2-wk interval. Two weeks later, animals were challenged contralaterally into the back by s.c. implantation of 5×10^5 Pmel17/gp100$^+$ M3-7 melanoma cells. Although about 50% of DBA/2 mice immunized with CpG-enriched pMCG16/mPmel17 pDNA were protected against a subsequent challenge with Pmel17/gp100$^+$ M3-7 melanoma cells, only about 18% of animals vaccinated with non-modified pUK21-A2/mPmel17 pDNA showed complete protection ($p < 0.01$) (*A*). Control animals—injected with either vector alone (empty pMCG16 or pUK21-A2 pDNA) according to the protocol described above—consistently failed to reject Pmel17/gp100$^+$ M3-7 melanoma cells (*B*).

nized with CpG-enriched pMCG16/mPmel17 pDNA were completely protected against the highly tumorigenic dose of 5×10^5 Pmel17/gp100$^+$ M3-7 cells (pooled results of two sets of experiments). None of the protected animals developed a tumor at a later time point during the entire observation period of 4 mo (Fig 2*A*). By contrast, injection with pUK21-A2/mPmel17 pDNA prevented the growth of the M3-7 inoculum in only four of 22 mice (18%, Fig 2*A*, $p < 0.01$). In sharp contrast, control animals that had been injected with either vector only (empty vectors) consistently failed to reject Pmel17/gp100$^+$ M3-7 melanoma cells ($p < 0.001$ for both pairs, Fig 2*B*).

The protective anti-tumor host response triggered by CpG motifs is antigen-specific Mice received two intradermal injections of the parental pUK21-A2/mPmel17 construct, its CpG-enriched variant pMCG16/mPmel17, or either vector only and were subsequently challenged with

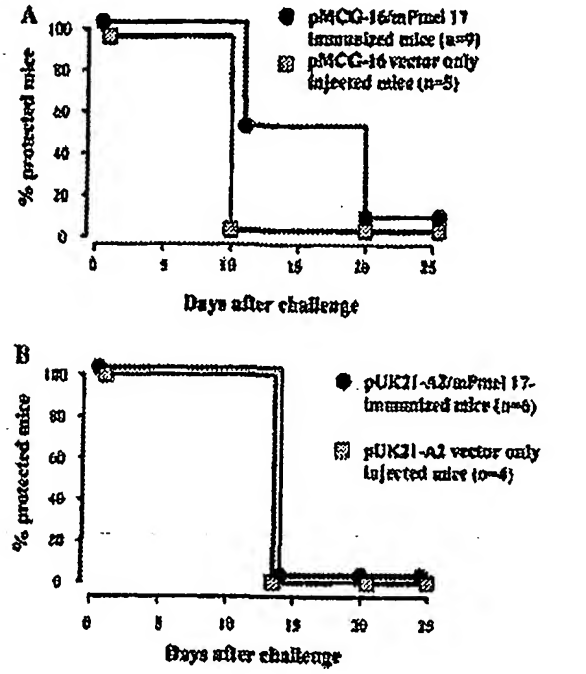

Figure 3
Administration of CpG-enriched pMCG16 plasmid-based DNA (pDNA) coding for Pmel17 protects animals against the growth of melanoma cells in an antigen-specific manner. Mice were injected with pMCG16/mPmel17 pDNA twice and subsequently challenged with Pmel17/gp100$^-$ M3-1 melanoma cells according to the protocol described under Materials and Methods. In contrast to the protection rates observed against a challenge with Pmel17/gp100$^+$ M3-7 melanoma cells (Fig 2*A*), none of the animals was able to reject Pmel17/gp100$^-$ M3-1 melanoma cells nor were control-treated mice injected with empty pMCG16 pDNA alone (*A*). The same observation was made for mice immunized with non-enriched pUK21-A2/mPmel17 pDNA and the animals injected with the respective control pUK21-A2 vector pDNA alone when challenged with Pmel17/gp100$^-$ M3-1 melanoma cells (*B*).

5×10^5 Pmel17/gp100$^-$ M3-1 melanoma cells. In contrast to the protection observed against the Pmel17/gp100$^+$ M3-7 cells as a function of CpG content of mPmel17-encoding pDNA vectors, neither pUK21-A2/mPmel17 (six of six animals) nor pMCG16/mPmel17 pDNA (nine of nine animals) were able to protect the animals against a challenge with antigen-negative M3-1 melanoma cells (Fig 3*A*, *B*). All animals developed tumors as did the vector-only injected (Fig 3*A*, *B*) or naïve control mice.

Antigenic specificity of the protective immunity induced by CpG motifs in pDNA-based vaccines was addressed in a further set of experiments. Animals that had developed protective anti-tumor immunity after vaccination with CpG-enriched pMCG16/mPmel17 pDNA, as demonstrated by successful rejection of Pmel17/gp100$^+$ M3-7 cells, were challenged with syngeneic but histogenetically unrelated Pmel17/gp100$^-$ P815 mastocytoma cells. Similar to non-immunized control mice, none of these animals (zero of six) was able to reject this tumor cell inoculum (Fig 4). Taken together, these results indicate that the protective anti-tumor host response induced by CpG-enriched pMCG16/mPmel17 pDNA is antigen-specific.

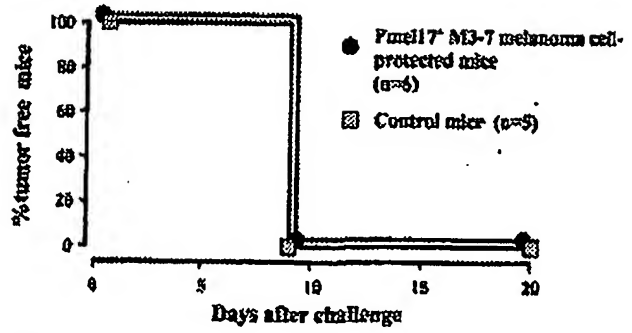

Figure 4
Growth characteristics of syngeneic Pmel17/gp100⁻ P815 mastocytoma cells. Mice were injected with pMCG16/mPmel17 plasmid-based DNA (pDNA) twice and subsequently challenged with Pmel17/gp100⁺ M3-7 melanoma cells according to the protocol described under Materials and Methods. Mice that had successfully rejected this melanoma cell inoculum were challenged 8 wk later by the s.c. inoculation of 1×10^5 syngeneic Pmel17/gp100⁻ P815 mastocytoma cells. Note that animals consistently developed tumors upon injection of Pmel17/gp100⁻ P815 cells. These tumors grew at a rate comparable with that observed in control mice injected with empty pMCG16 pDNA (control mice).

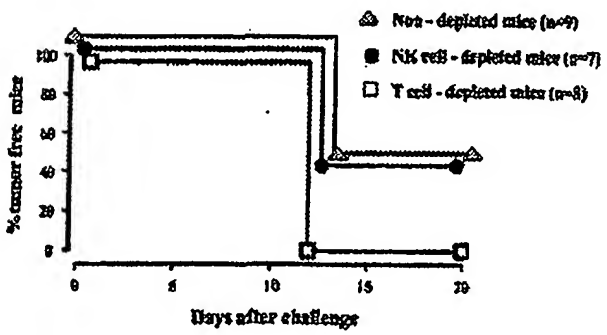

Figure 5
Tumor cell protection is mediated by T cells but not NK cells. Mice were injected with pMCG16/mPmel17 pDNA twice in a 2-wk interval and challenged 14 d later with Pmel17/gp100⁺ M3-7 melanoma cells. To deplete T cells, animals received i.p. injections of anti-CD4 (0.3 mg GK1.5) and anti-CD8 (0.3 mg YTS169) antibody solutions at days −3, −2, and −1 before tumor cell challenge. To deplete NK cells, mice were injected i.p. on days −1, +1, 6, 11, 16, 21, 26, and 31 around tumor cell challenge with 200 μL of a 1:10 PBS dilution of a reconstituted anti-asialo-GM1 preparation. Animals were monitored for the appearance and growth of tumors as described above. Note complete loss of tumor protection in mice depleted of CD4⁺ and CD8⁺ T cells, but not in mice depleted of NK cells.

The protective anti-tumor effect triggered by CpG motifs requires the adaptive arm of the immune system To characterize the effector mechanisms triggered by CpG motifs present in pDNA-based vaccines, we performed T cell- and NK cell-depletion experiments. Animals that had received two injections of CpG-enriched pMCG16/mPmel17 were depleted of either CD4⁺/CD8⁺ T lymphocytes or NK cells before being challenged with Pmel17/gp100⁺ M3-7 melanoma cells. In this experiment, eight of eight CD4⁺/CD8⁺ T cell-depleted animals developed tumors, indicating that the protective anti-tumor effect otherwise induced by the CpG-enriched pDNA construct was completely dependent on the presence of T lymphocytes (Fig 5). In contrast, NK cell depletion resulted in only a minor, if any, loss of the animals' ability to reject Pmel17/gp100⁺ M3-7 melanoma cells (three protected of seven immunized animals) as compared with non-NK cell-depleted, pMCG16/mPmel17-immunized animals (four protected of nine immunized animals, Fig 5). Taken together, these results demonstrate that mainly T cells represent the critical effector population of protective anti-tumor host responses elicited by application of CpG-enriched antigen-specific pDNA constructs.

To confirm the stimulatory effect of CpG motifs in pDNA-based vaccines on the adaptive arm of the immune system more directly, CTL assays were performed. Splenocytes from pUK21-2A/mPmel17 and pMCG16/mPmel17 immunized as well as vector-only injected animals were tested for the presence of Pmel17/gp100-specific CTL. Spleen cells of animals injected with the CpG-enriched pMCG16/mPmel17 pDNA exhibited significant lysis of Pmel17/gp100⁺ M3-7 target cells whereas they failed to kill Pmel17/gp100⁻ M3-1 cells (Fig 6*A*). By contrast, neither M3-7 nor M3-1 cells were significantly lyzed by spleen cells from naive control animals (Fig 6*B*). YAC-1 lysis at significant levels was not detected in these assays (Fig 6*C*), which excluded a significant contribution of NK cell activity on killing of M3-7 melanoma cells. Together with the demonstration that both M3-7 and M3-1 cells are similarly susceptible to the cytotoxic activity of lymphokine-activated killer (LAK) cells as well as allo-specific CTL (data not shown), these results suggest that killing of Pmel17/gp100⁺ M3-7 cells obtained with splenocytes from animals immunized with CpG-enriched pMCG16/mPmel17 pDNA is executed by Pmel17/gp100-specific T lymphocytes.

In these CTL assays, splenocytes from animals immunized with CpG-enriched pMCG16/mPmel17 pDNA were consistently found to exhibit a more pronounced (1.5–2 titer levels) antigen-specific CTL activity than splenocytes from animals injected with pUK21-2A/mPmel17 pDNA (see Fig 6*A, D*). To further confirm this observation, we performed genetic vaccination with pUK21-A2/β-galactosidase and pMCG16/β-galactosidase pDNA vectors according to the protocol described above. Induced antigen-specific T cell numbers were quantitatively evaluated with the help of ELISPOT assays using the β-galactosidase encoded, $H2^d$-restricted immunodominant peptide TPHPARIGL. In these assays, splenocytes derived from mice immunized with CpG-enriched pMCG16 pDNA vector again exhibited reproducibly higher frequencies of antigen-specific IFN-γ-secreting T cells than splenocytes derived from mice immunized with the parental pDNA vector pUK21-2A (Fig 7).

Discussion

There are several strategies to enhance the potential of pDNA vaccines for the induction of an effective IR. These strategies include the facilitation of pDNA uptake into and release within targeted cells, e.g. by complexing with polycations/lipopolyplexes or electroporation, enhancement of the nuclear association of transgenes or targeting

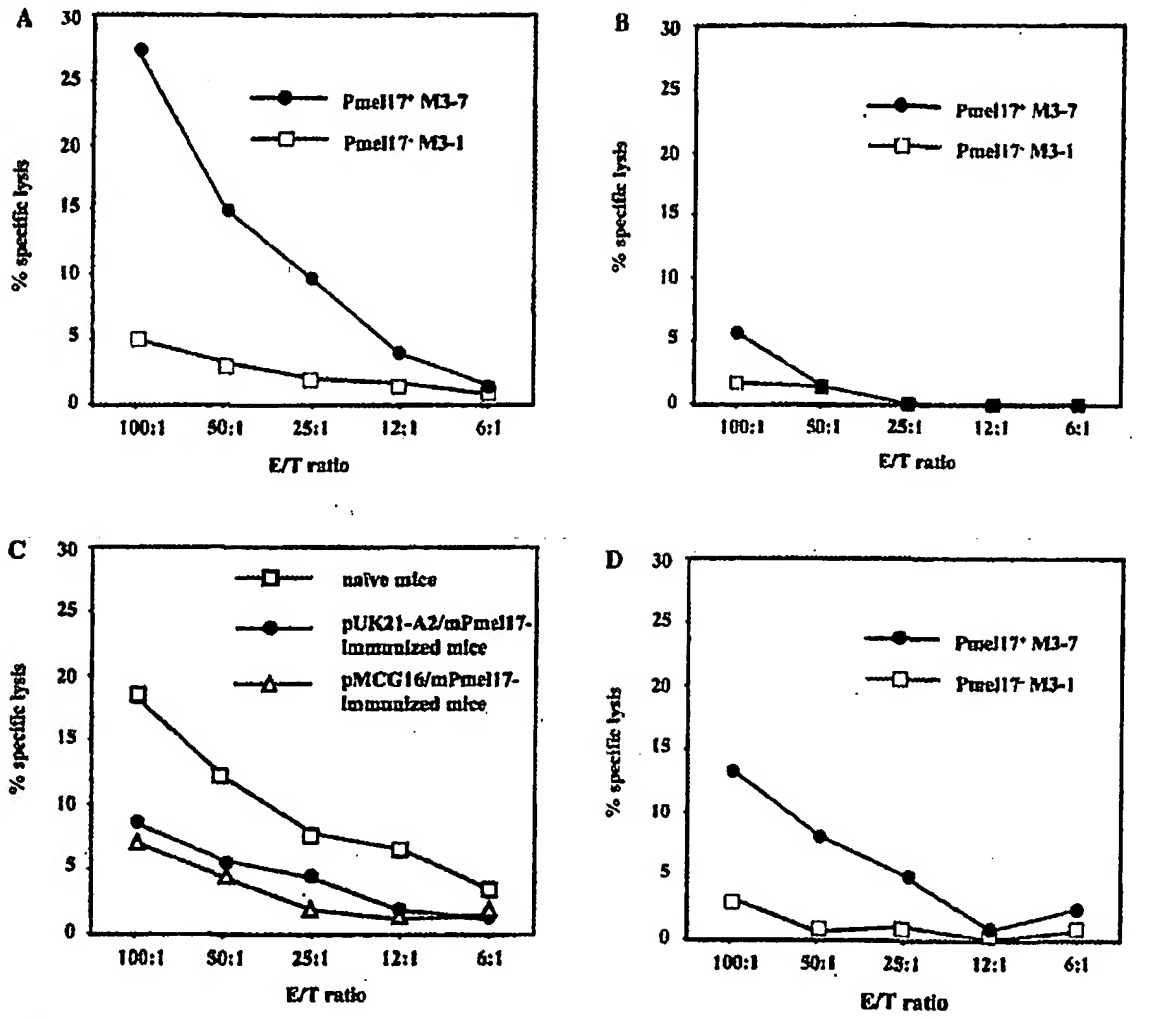

Figure 6
Intracutaneous genetic immunization with CpG-enriched pMCG16/mPmel17 plasmid-based DNA (pDNA) results in the generation of an antigen-specific CTL response. Splenocytes, recovered 2 wk after the second immunization from pUK21-A2/mPmel17, pMCG16/mPmel17 recipients, and naïve control mice, respectively, were restimulated for 4 d with interferon (IFN)-γ- and PFA-treated Pmel17/gp100$^+$ M3-7 cells. Lysis of target cells mediated by restimulated splenocytes was determined in a 6 h standard ^{51}Cr-release assay. Target cells included Pmel17/gp100$^+$ M3-7, Pmel17/gp100$^-$ M3-1 melanoma cells (both pre-stimulated with 100 U per mL IFN-γ for 18 h) and YAC-1 cells. In these assays, splenocytes from mice immunized with CpG-enriched pMCG16/mPmel17 pDNA lysed Pmel17/gp100$^+$ M3-7 melanoma cells at significant levels whereas Pmel17/gp100$^-$ M3-1 melanoma cells were not lysed (*A*). Neither M3-7 nor M3-1 melanoma cells were lysed by splenocytes derived from naïve mice (*B*). NK cell target cells YAC-1 were lysed at irrelevant levels by splenocytes derived from mice injected with CpG-enriched pMCG16/mPmel17 pDNA as well as pUK21-A2/mPmel17 pDNA (*C*). CTL activity of splenocytes derived from animals injected with pUK21-2A/mPmel17 pDNA (*D*) recorded consistently lower levels (1.5–2 titer levels) of Pmel17/gp100$^+$ M3-7 lysis when compared with levels obtained with splenocytes from animals injected with CpG-enriched pMCG16/mPmel17 pDNA (*A*). Standard deviations of triplicates were <5% and have been omitted from figures for clarity.

to antigen-presentation pathways, modification to antigens, and increase in immunogenicity by use of adjuvants (overview in Donnelly *et al*, 2003; Herweijer and Wolff, 2003). For the latter objective, the classical rules of application of adjuvants to proteins are not transferable to DNA vaccines due to significant differences of antigen expression in terms of time kinetics and quantitative levels obtained. Thus, many adjuvants effective in protein-based immunization strategies are not or not as effective for DNA vaccines (reviewed in Spack and Sorgi, 2001). Consequently, identification of adjuvants augmenting the activity of pDNA vaccines is a major challenge for the successful development of DNA vaccines.

Replication-deficient viral vectors, particularly adenoviral vectors, can induce impressive specific cellular IR by taking advantage of their recognition as pathogen-associated molecular patterns (PAMP) through TLR expressed on/in cells of the innate immune system. But the frequent generation of impressive memory responses against the vector itself considerably inhibits the boost of an IR against the encoded transgene by repetetive application (St George, 2003). Among the many types of PAMP, immunostimulatory DNA motifs have been shown to interact specifically with TLR9, which is expressed in CD123$^+$ pDC (Bauer *et al*, 2001a; Bauer *et al*, 2001b), a key regulatory cell for the activation not only of innate but also

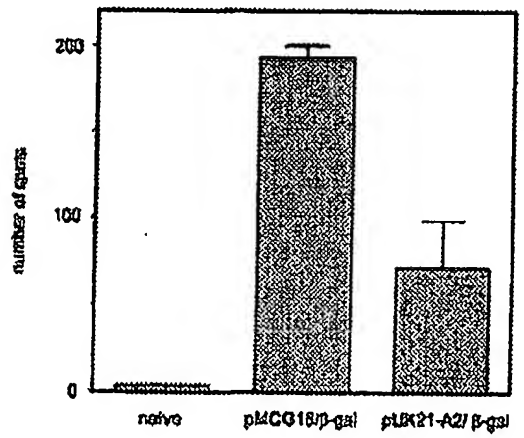

Figure 7
Induction of antigen specific T cell responses as measured by ELISPOT assay. Mice were immunized with pUK21-A2/β-galactosidase or pMCG16/β-galactosidase plasmid-based DNA (pDNA) according to the protocol described for the mPmel17-containing pDNA constructs. Ten days after the second injection, splenocytes were collected from immunized recipients as well as from naïve control mice and restimulated with an H-2^d-restricted β-galactosidase peptide TPHPARIGL overnight. In this assay, splenocytes from mice immunized with CpG-enriched pMCG16/β-galactosidase pDNA (pMCG16/β-gal) exhibited consistently higher frequencies of IFN-γ-secreting T cells than those derived from mice immunized with parental unmodified pUK21-A2/β-galactosidase pDNA (pUK21-A2/β-gal). Background levels were observed in splenocytes from naïve animals (naïve). Values are given for 0.25×10^6 splenocytes.

of adaptive IR (Arpinati *et al*, 2003). In this study, we now demonstrate that modification of the backbone sequence of a pDNA vaccine for the presence of this subgroup of PAMP results (i) in a significantly increased protection against a subsequent, otherwise lethal, challenge with antigen-positive, but not antigen-negative syngeneic melanoma cells, (ii) in the induction of a protective T cell but not NK cell response *in vivo* as demonstrated by *in vivo* cell depletion assays, (iii) in the detection of an increased and antigen-dependent target cell lysis by T cells but not NK cells *in vitro*, and (iv) in the detection of increased numbers of antigen-specific, IFN-γ-secreting T cells.

Anti-tumor effects of immunostimulatory CpG motifs, particularly in the form of CpG ODN, have been described against various tumor types when applied with or without antigen or antibodies (Carpentier *et al*, 2000; Lanuti *et al*, 2000; Ballas *et al*, 2001; Kawarada *et al*, 2001; Miconnet *et al*, 2002; Zwaveling *et al*, 2002; Baines and Celis, 2003; Baral *et al*, 2003; Davila *et al*, 2003; Sandler *et al*, 2003). This may be the result of the induction and/or enhancement of different effector mechanisms. In most of these models, the anti-tumor effect of CpG motifs was mediated via activation of the innate immune system directly through NK cells, but enhancement of ADCC (Wooldridge *et al*, 1997) and, more recently, induction of adaptive cellular IR have also been described (Ballas *et al*, 2001; Miconnet *et al*, 2002; Zwaveling *et al*, 2002; Baines and Celis, 2003; Davila *et al*, 2003). Using the B16/C57BL/6 melanoma model, Ballas *et al* (2001) described induction of anti-tumor immunity with regression of established melanomas by application of CpG ODN 1585. The protection of TAP-1- and β2-microglobulin-knockout mice to a degree similar to normal C57BL/6, lack of immunological memory in surviving animals, and loss of protection after *in vivo* NK cell depletion strongly suggest the stimulation of an NK cell-mediated effector mechanism. Several of our findings make it very unlikely that this effector mechanism is also operative in the M3/DBA/2 mouse melanoma model. These include the lack of abrogation of protective effects in NK cell depletion experiments, the lack of significant NK cell activity in CTL assays, and the inability of mice injected with the CpG-enriched vector-only construct to reject melanoma cells. In contrast, the protection against antigen-positive, but not antigen-negative melanoma cells, the loss of anti-tumor protection by *in vivo* T cell depletion assays, and the presence of antigen-specific CTL indicate the stimulation of antigen-specific T cell-mediated immunity. These observations are in agreement with the results described in the B16/C57BL/6 melanoma model and the transgenic HHD mouse model when using CpG motifs, namely CpG ODN 1826, along with melanoma antigens provided as peptides (Miconnet *et al*, 2002; Davila *et al*, 2003). In combination immunotherapy with an antibody blocking the interaction of CTLA-4 on T cells with its ligands B7.1 and B7.2, which may provide negative signals to T cells, application of CpG ODN plus MDA $TRP2_{180-188}$ CTL epitope induced therapeutic immunity that required both $CD4^+$ and $CD8^+$ T cells (Davila *et al*, 2003). Furthermore, human human D^b mice, which are transgenic for chimeric human HLA-A*0201/mouse D^b MHC class I molecules, mounted antigen-specific and cytolytic $CD8^+$ T cell responses after application of a peptide analog of human MDA MART-1/Melan-A_{26-35} in the presence of CpG ODN 1826 (Miconnet *et al*, 2002). The different kinds of IR triggered by CpG motifs in these melanoma models may be due to the different genetic background of the animal models used; however, there may be additional explanations including the different routes of application of the compounds (i.p. *vs* s.c.) (Cohen *et al*, 1998) and, most importantly, the type of CpG motif used. CpG ODN 1826 and 1585 significantly differ in their ability to induce cytokine secretion and NK cell activation in C57BL/6 splenocytes. Although CpG ODN 1826 induces high levels of interleukin (IL)-6, IL-12, IFN-γ, and tumor necrosis factor-α, a cytokine pattern that may favor the induction of Th1-biased T cell responses, CpG ODN 1585 is a much more potent inducer of NK cell activity (Ballas *et al*, 2001). These differential immunostimulatory properties are now known to represent a direct function of sequence composition and backbone modification of ODN (Krieg, 2002).

Several observations derived from systems employing model antigens (Mor *et al*, 1995; Sato *et al*, 1996; Tighe *et al*, 1998) have supported the view that increasing the number of CpG motifs in pDNA may indeed result in the induction of more effective cellular IR. Our results obtained with the model antigen β-galactosidase are in accordance to those described by Sato *et al* (1996), who demonstrated that application of a β-galactosidase-encoding vector modified for its selectable marker, i.e. substitution of the CpG-poor kanamycinR gene by the CpG-rich ampicillinR gene, results in a much more efficient induction of antigen-specific T cell responses. Our results support these data and extend them by two further observations: (i) the significant adjuvant

effect on the induction of T cell activity against a weakly immunogenic (non-model, non-viral) self-antigen and (ii) the translation of this IR into enhanced protective cancer immunity. Even though pDNA vectors cannot be directly compared with CpG ODN for the immunostimulatory effects induced, the immunological phenomena described in this study support the assumption that the backbone modifications of the pDNA vector have resulted in an adjuvant effect comparable with that described for CpG ODN 1826. This study extends our knowledge on these immunostimulatory properties of antigen-specific pDNA vaccines and supports the concept of enhancing the immunogenicity of DNA vaccines by modification with PAMP.

Materials and Methods

Mice Female 6–10-wk-old DBA/2 (H-2^d) mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) and held under specific pathogen-reduced conditions in the Central Animal Laboratory (University of Essen, Germany). All animal experiments were approved by the regulatory committee on animal welfare in the state of Northrhine-Westfalia and the Austrian Ministry of Science and Transportation.

Cell lines Clone M3 (derived from Cloudman S91 melanoma; H-2^d) (Yasumura *et al*, 1966), P815 mouse mastocytoma (H-2^d), YAC-1, and COS-7 cells were obtained originally from the American Type Culture Collection (ATCC) (Rockville, Maryland). M3 melanoma cells were cultured in Ham's F10 medium (Life Technologies, Grand Island, New York) supplemented with 2.5% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Eggenstein, Germany), 12.5% horse serum (Life Technologies), 0.1 mM non-essential amino acids (Gibco BRL), 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5×10^{-5} M 2-ME (Life Technologies) in gelatin-coated culture flasks (Becton Dickinson, Heidelberg, Germany). The generation of M3 sublines, Pmel17/gp100$^+$ M3-7 (>95% of cells immunoreactive with Pmel17/gp100-reactive mAb HMB45), and Pmel17/gp100$^-$ M3-1 cells (<5% of cells immunoreactive with mAb HMB45) has been described previously (Wagner *et al*, 2000). Pmel17/gp100 expression of these cells was regularly checked by FACS analysis or immunostaining of cytospin preparations. YAC-1 and P815 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS, HEPES, non-essential amino acids, L-glutamine, sodium pyruvate, and 2-ME. COS-7 cells were grown in DMEM supplemented with 10% FCS. All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Construction of pDNA vectors and *in vitro* transcription/translation analysis of introduced transgenes The pDNA vector pUK21-A2 contains the immediate early promoter of human cytomegalovirus (CMV IE), the bovine growth hormone polyadenylation signal, and the kanamycin resistance gene (Krieg *et al*, 1998). The vector backbone of pUK21-A2 was modified by eliminating 52 of 134 neutralizing CpG (-N) and addition of 16 immunostimulatory CpG (-S) motifs, thereby generating its variant pDNA vector pMCG16. Briefly, site-directed mutagenesis by overlap-extension PCR (Prosch *et al*, 1996) was employed to introduce a total of 22 point mutations into the kanamycin resistance gene and non-essential DNA backbone sequences after the gene. Fifteen CpG-N motifs containing 19 CpG dinucleotides were modified, 12 of which were eliminated and seven of which were transformed into CpG-S motifs. All the mutations were confirmed by sequencing. Another 37 CpG-N motifs were removed by replacing a 0.6 kb *NarI–EcoO*109I fragment containing the F1 origin with an artificial multiple cloning site consisting of a 35 bp double-stranded DNA fragment containing four unique restriction enzyme sites (*DraI*, *ScaI*, *AvaII*, *HpaI*) (Krieg *et al*, 1998). Additional nine CpG-S motifs were inserted into the vector by self-ligation of a 20 bp DNA fragment (5′-GACTCCATGACGTTCCTGACGTTTCCA TGACGTTCCTGACGTTG-3′) with a complementary strand and insertion of these copies into the *AvaII* site of the vector. Vector sequences have been deposited in GenBank under accession numbers AF053408 and AF053406.

pUK21-A2/mPmel17 and pMCG16/mPmel17 pDNA were generated by ligation of the 1.9 kb murine full-length Pmel17 cDNA sequence (Schreurs *et al*, 1997; Zhai *et al*, 1997) into the respective *NotI* cloning sites of pUK21-A2 and pMCG16 vectors. Full-length pUK21-A2/β-galactosidase and pMCG16/β-galactosidase vectors (MacGregor and Caskey, 1989) and pUK21-A2/EGFP and pMCG16/EGFP encoding EGFP were generated accordingly. Correct orientation and sequence of the respective transgenes were confirmed by automated sequencing on an ABI PRISM genetic analyzer (PE Biosystems, Weiterstadt, Germany) or restriction enzyme mapping (EGFP). All pDNA constructs were affinity purified with the Endo-free Plasmid Mega Kit (Qiagen, Hilden, Germany) and all pDNA constructs contained <0.04 IU per μg of endotoxin, as determined by the Limulus Amoebocyte Lysate Assay kit (QCL-1000, BioWhittaker, Walkersville, Maryland).

Two micrograms of each vector construct was transfected into 50% confluent COS-7 cells using the DEAE dextran/chloroquine diphosphate method essentially as described (Wagner *et al*, 1995). After 48 h of incubation, transgene expression was assessed either by FACS analysis (EGFP, Coulter-Immunotech, Krefeld, Germany), immunocytochemistry with anti-Pmel17/gp100 Ab HMB45, or enzymatic cleavage of 5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside (X-Gal) for β-galactosidase activity.

Genetic vaccination and tumor challenge DBA/2 mice were injected intradermally on one side of the shaved back with 100 μg of pUK21-A2/mPmel17 and pMCG16/mPmel17 pDNA constructs, twice in a 2-wk interval. Control animals received 100 μg vector pDNA alone. Two weeks later, animals were challenged contralaterally into the back by s.c. implantation of 5×10^5 Pmel17/gp100$^+$ M3-7 or Pmel17/gp100$^-$ M3-1 cells. Tumor growth was monitored every 2–3 d. All *in vivo* experiments were read in a blinded, randomized fashion.

In an additional set of experiments, Pmel17-immunized animals that had successfully rejected a M3-7 melanoma inoculum were challenged 8 wk after implantation of M3-7 cells with the s.c. inoculation of 1×10^5 syngeneic P815 mastocytoma cells.

***In vivo* cell depletion** To deplete T cells, animals received i.p. injections of anti-CD4 (0.3 mg GK1.5) and anti-CD8 (0.3 mg YTS169) antibody solutions at days −3, −2, −1 before the tumor cell challenge as described (Luhrs *et al*, 2002). This treatment led to a >95% reduction of splenic CD3-positive cells as determined by FACS analysis (not shown). Fourteen days after the second vaccination, mice were inoculated s.c. with 5×10^5 Pmel17/gp100$^+$ M3-7 melanoma cells and monitored for the appearance and growth of tumors as described above. A rabbit anti-asialo-GM1 Ig preparation (Wako Chemicals, Neuss, Germany) was used to deplete mice of NK cells as described (Schneeberger *et al*, 1998). Mice were injected i.p. on days −1, +1, 6, 11, 16, 21, 26, and 31 around tumor cell challenge with 200 μL of a 1:10 phosphate-buffered saline (PBS) dilution of the reconstituted anti-asialo-GM1 preparation. This treatment led to a >90% reduction of the splenic NK cell activity (as assumed by lysis of the NK cell target YAC-1) as compared with spleen cells of non-treated mice or mice injected with a dialyzed 1:10 dilution of normal rabbit serum (ICN Pharmaceuticals, Costa Mesa, California) in PBS.

Cytotoxicity assay Splenocytes (4×10^6 per well), recovered 2 wk after the second immunization from pUK21-A2/mPmel17 and pMCG16/mPmel17 recipients or from naïve control mice, were co-cultured for 4 d with 1×10^5 IFN-γ (200 U per mL for 18 h; Genzyme, Framingham, Massachusetts)-stimulated and PFA-treated (0.5% PFA for 10 min at 37°C) M3-7 cells as described

(Wagner *et al*, 2000). Recombinant human (rh) IL-2 (Cetus Corp., Emeryville, California) was added at a final concentration of 10 U per mL. Lysis of target cells mediated by restimulated splenocytes was determined in a 5 h standard ^{51}Cr-release assay. For this purpose, 5×10^3 Na_2[^{51}CrO4 (ICN, Eschwege, Germany)-labeled target cells were incubated with splenocytes at various effector-to-target ratios in triplicate U-bottomed 96-well plates (Greiner, Solingen, Germany) in a final volume of 200 μL. ^{51}Cr released from the targets into the supernatant was quantified in a liquid scintillation counter (Beckman LS-6000, Munich, Germany). Target cells included Pmel17/gp100$^+$ M3-7 and Pmel17/gp100$^-$ M3-1 melanoma cells (both pre-stimulated with 100 U per mL IFN-γ for 18 h) and YAC-1 cells used as control for NK cell activity within the spleen cell populations. To generate LAK cells, splenocytes of naïve animals were cultured for 5 d in complete medium supplemented with 200 U per mL rhIL-2 (Cetus Corp.). The percentage of specific lysis was calculated as [(sample c.p.m.−spontaneous c.p.m./(maximal c.p.m.−spontaneous c.p.m.)] × 100. Spontaneous release in all cases ranged between 10% and 20% of the maximum release obtained by treatment of labeled target cells with 1% Triton X-100 (Sigma, Deisenhofen, Germany).

ELISPOT assay Mice were immunized intracutaneously two times in 2 wk interval with pUK21-A2/β-galactosidase or pMCG16/β-galactosidase pDNA according to the protocol described for the mPmel17-containing pDNA constructs. Ten days later, splenocytes were collected from immunized recipients as well as from naïve control mice. Multiscreen 96-well assay plates (Millipore, Bredford, Massachusetts) were pre-coated overnight at 4°C with 5 μg per mL anti-IFN-γ antibody R4-6A2 (Schmitt *et al*, 1994) in 0.1 M sodium carbonate buffer pH 9.3. After washing with PBS/0.05% Tween-20, plates were blocked for 2 h with DMEM standard medium (Gibco BRL). Splenocytes were plated in duplicate at a density of 2×10^6 per 100 μL in DMEM standard medium. For restimulation, 100 μL of the H-2^d-restricted β-galactosidase peptide (TPHPARIGL) at a final concentration of 20 μg per mL was added. Plates were cultured overnight at 37°C, 5% CO_2, washed extensively, and incubated with 100 μL of the biothylated anti-IFN-γ mAb AN18.17.24 (2 μg per mL; Schmitt *et al*, 1994) for 2 h. Detection was carried out with peroxidase conjugated to streptavidin at a dilution of 1/5000 (Boehringer Mannheim, Mannheim, Germany). After 1 h of incubation with peroxidase-conjugated streptavidin (dilution of 1:5000, Boehringer Mannheim), spots were developed by adding 100 μL of substrate (0.8 mg per mL 3,3′-Diaminobenzidine (Sigma)/0.4 mg per mL $NiCl_2$ (Sigma)/0.009% H_2O_2 in 0.1 M Tris pH 7.5) and the reaction was stopped after 10–30 min by washing with deionized H_2O. Spots were counted subsequently with the ELISPOT Bioreader-2000 (Biosys GmbH, Karben, Germany).

Statistical analysis Fisher's exact test was used to evaluate statistical differences in tumor protection rates between pUK21-A2/mPmel17 and pMCG16/mPmel17-immunized mice as well as to evaluate differences with the respective control vector-treated mice.

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